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IN VITRO AND ANIMAL STUDIES



Lactobacillus plantarum 22A-3 exerts anti-allergic activity through TGF- β secretion in passive cutaneous anaphylaxis of mice

Minagi Fujii^a, Ko Fukuura^a, Nobuaki Ohto^b, Hiroshige Kuwahara^b and Masashi Mizuno^a

^aDepartment of Agrobioscience, Graduate School of Agricultural Science, Kobe University, Kobe, Japan; ^bMaruzen Pharmaceuticals Co. Ltd., Fukuyama, Japan

ABSTRACT

Allergy is a global issue, however, medical intervention for allergy treatment is limited. Recent studies have focussed on allergy prevention with food factors. In this study, *Lactobacillus plantarum* 22A-3 (LP22A3) exerted an anti-allergic effect in passive cutaneous anaphylaxis (PCA) reaction and increased transforming growth factor (TGF)- β contents in blood. The increase of TGF- β contents in blood by exogenous TGF- β injection intraperitoneally decreased Evans blue release into mice ears to the same level as LP22A3 treatment in PCA reaction. LP22A3 treatment directly to RBL-2H3 cells shows no effect on β -hexosaminidase release from RBL-2H3 but inhibited its release using the Caco-2/RBL-2H3 cells co-culture system stimulated with LP22A3 from the apical side. Moreover, TGF- β treatment to RBL-2H3 inhibited β -hexosaminidase release from RBL-2H3. However, β -hexosaminidase release was cancelled by TGF- β neutralising antibody without the influence of TGF- β mRNA expression in Caco-2 cells. These results showed that LP22A3 ameliorates allergy by TGF- β secretion through the intestine.

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Introduction

Allergic disease is caused by hypersensitivity of the immune system. Hypersensitivity can be divided into four types, namely: immediate hypersensitivity (type I), cytotoxic reaction (type II), immune complex reaction (type III), and delayed hypersensitivity (type IV) (Janeway et al. 2001). Type I is initiated by the allergen, including food factors, pollens, specks of dust, etc. When allergen gets access to the human body, it will be recognised by antigen-presenting cells (APC) and presented to naïve helper T cell (Th0). Th0 is activated and differentiates into T helper 2 cell (Th2) rather than T helper 1 cell (Th1). Th2 is capable of secreting Th2 cytokines including Interleukin-4 (IL-4), IL-5 and IL-13, which can promote B cell to process immunoglobulin (Ig) class switch recombination and leading to the accumulation of IgE. When the same allergen invades again, the binding of allergen to IgE activates the Fc ϵ Receptor I (Fc ϵ RI) on mast cells and causes mast cells degranulation resulting in the release of chemical mediators like histamine (Janeway et al. 2001). Effects induced by histamine contain smooth muscle contraction, increased vascular permeability, prostaglandin generation (White 1990),

which will lead to symptoms like skin reactions (urticarial, eczema and angioedema), respiratory tract reactions (rhinitis and bronchitis), gastrointestinal tract (intestinal cramps and diarrhea) and the worst, anaphylactic shock (Janeway et al. 2001).

Transforming growth factor (TGF)- β is a cytokine that regulates cell differentiation, migration, and proliferation, and TGF- β plays a crucial role in maintaining skin homeostasis (Ramirez et al. 2014). It is well known that TGF- β promotes collagen production in the skin (Hwang et al. 2011; Hwang et al. 2014), and moreover, TGF- β is known to promote keratinocyte migration, which is essential for the reconstruction of the cutaneous barrier after skin injury (Zambruno et al. 1995; Santoro and Gaudino 2005). It has been reported that TGF- β acts as a negative regulator of mast cell function, in part by decreasing Fc ϵ RI expression (Gomez et al. 2005). However, it is not well understood whether TGF- β affects allergic properties.

Lactobacillus plantarum 22A-3 (LP22A3) is one of the plants derived lactic acid bacteria (LAB) and it was found in the rice-bran paste of eggplants pickle. A previous study indicated that its oral administration prevented DSS-induced colitis (Ohto and Mizuno 2017).

Moreover, it was demonstrated that anti-inflammatory cytokine, TGF- β and IL-10 productions were increased from lamina propria lymphocytes by oral administration of LP22A3 to the normal mice. This report also indicated that LP22A treatment in the apical side of Caco-2/RAW264.7 cells co-culture system cancelled the inhibition of IL-8 mRNA expression of Caco-2 cells when TGF- β neutralising antibody was added to the basolateral side before stimulating RAW264.7 cells by lipopolysaccharide. Overexpression of TGF- β abolished the airway hyperresponsiveness and airway inflammation in the murine model of allergic asthma (Hansen et al. 2000). Administration of anti-TGF- β antibody to sensitised mice before the transfer of CD4⁺CD25⁺ T regulatory cells countered the suppression effect of these cells on allergen-induced airway hyperresponsiveness (Joetham et al. 2007). Moreover, Jiménez et al. (2016) reported that the oral administration of glycomacropeptide prior to and during sensitisation increases the production of TGF- β in response to allergens. Our previous study has demonstrated that oral administration of *Enterococcus faecalis* IC-1 prevents allergic symptoms in passive cutaneous anaphylaxis (PCA) reaction and Caco-2/RBL-2H3 co-culture system (Yamashita et al. 2016).

The aim of this study was to investigate whether oral administration of LP22A3 can regulate mast cell activation by PCA and co-culture system, so as to understand the mechanism by TGF- β production. It was ascertained to the practical application of LP22A3 for the control of allergic diseases.

Materials and methods

Materials

Dulbecco's Modified Eagle's Medium (DMEM, High Glucose) with glutamine and Evans blue were purchased from Wako Pure Chemical Industries (Osaka, Japan). Eagle's Minimum Essential Medium (MEM) was purchased from Nissui Pharmaceutical Co. Ltd., (Tokyo, Japan). Anti-dinitrophenyl (DNP) IgE, DNP-albumin, and *p*-nitrophenyl *N*-acetyl- β -D-glucosaminide were purchased from Sigma (St. Louis, MO). Trypsin, RPMI 1640 medium, MEM non-essential amino acids (NEAA) were purchased from Gibco BRL (Grand Island, NY). Foetal bovine serum (FBS) was purchased from Thermo Fisher Scientific (Waltham, MA). DNP-bovine serum albumin (DNP-BSA) was purchased from Cosmo Bio (Tokyo, Japan). Recombinant mouse TGF- β was purchased from BioLegend (San Diego, CA). Mouse anti-TGF- β antibody was purchased from an R&D system (Minneapolis, MN). Other chemicals and reagents were ordinary commercial and guaranteed products.

Preparation of UV-inactivated LP22A3 and oral administration

LP22A3 was cultured in Mann Rogosa Sharp (MRS) broth and incubated overnight at 30 °C in an anaerobic chamber. After incubation, bacterial cells were obtained by centrifugation (4 °C, 10,000 $\times$ g, 5 min) and was resuspended in PBS and washed three times to remove the culture medium. The bacterial suspension was irradiated with a UV germicidal lamp to inactivate before use in *in vitro* experiments. After UV-treatment, viable counts were below 10² cfu/mL indicated more than 9-log reduction of viability. Samples were stored at -80 °C until use. The inactivated LP22A3 (1 $\times$ 10¹¹ cfu/g) was suspended in 0.5% carboxymethyl cellulose, and 100 μ L of it was orally administered using a gastric feeding tube.

Mice

Female 7-week-old BALB/c mice were purchased from Japan SLC (Shizuoka, Japan). Mice were maintained in filter-top cages in a specific pathogen-free condition in Kobe University Life Science Laboratory with free access to laboratory chow and water *ad libitum*. All animal experiments were approved and carried out in accordance with the Animal Experiment Ethics Committee of Kobe University (permission number: 30-10-03-R1).

PCA reaction in mice

An IgE-dependent PCA reaction was performed in accordance with a previous study (Yamashita et al. 2016). Mice were randomly divided into four groups with 4 animals in each group and were administered with 100 μ L of test sample orally for 10 days. Anti-DNP IgE (0.1 μ g) was injected subcutaneously into both ears of each mouse under anaesthesia on day 10. After 24 h, the mice were injected intravenously with 0.2 mg of DNP-BSA in 100 μ L PBS containing Evans blue (10 mg/mL), via tail vein. Thirty minutes after the DNP-BSA injection, mice were sacrificed and both ears were collected for measurement of the pigmented area. The ears were mixed in 250 μ L of formamide at 37 °C for 24 h. After centrifugation at 10,000 rpm for 20 min at 4 °C, the supernatants were used for measurement of absorbance at 620 nm. Luteolin was used as positive control. The recombinant TGF- β (17.5 ng/mouse) was intraperitoneally injected to increase TGF- β levels in blood every day for 10 days.

Cell culture

Rat basophilic leukaemia cell line, RBL-2H3 cells, were cultured in dishes in Eagle's MEM supplemented with 10% (v/v) heat-inactivated FBS (57 °C, 30 min), 100 µg/mL streptomycin, 100 U/mL penicillin, and 2 mM L-glutamine. Cell cultures were incubated at 37 °C in a 5% CO₂ incubator. Passage numbers 14–32 were used. Human intestinal epithelial cell line, Caco-2 cells, were cultured in a 75 cm² plastic flask in DMEM (high glucose) supplemented with 10% FBS, 1% MEM-NEAA, 100 µg/mL streptomycin, and 100 U/mL penicillin, and incubated at 37 °C in a 5% CO₂ incubator. Passage numbers 48–64 were used. When either cell line reached 80% confluence, cells were recovered from the culture dish or flask by trypsin digestion after washing with phosphate-buffered saline (PBS). The cells were replated in a new dish or flask.

Co-culture system of Caco-2/RBL-2H3 cells

Caco-2 cells were seeded at the concentration of 0.6×10^5 cells/well onto 24-well Transwell insert plates (0.33 cm², 0.4 µm pore size, Corning Co-star Corp., Cambridge, MA). The cell culture medium was changed every 3 days until the cells were fully differentiated (TER value >300 Ω cm²). RBL-2H3 cells were seeded at 2.0×10^5 cells/500 µL/well onto 24-well tissue culture plates in Eagle's MEM and incubated overnight with 1 µg/mL at a final concentration of anti-DNP IgE. After replacing all media with Siraganian buffer (SB; 119 mM NaCl, 5 mM KCl, 0.4 mM MgCl₂, 1 mM CaCl₂, 40 mM NaOH, 25 mM PIPES, 5.6 mM glucose, 0.1% BSA, pH 7.2) the Transwell inserts on which Caco-2 cells had been cultured were added into the plate wells preloaded with RBL-2H3 cells. In an experiment to evaluate the anti-allergic effect of test samples, 0.2 mL of SB or test sample solution was applied to the apical side. After incubation for 6 h, the cells were challenged with 10 ng/mL final concentration of DNP albumin for 10 min at 37 °C. The plate was cooled in an ice bath for 10 min to stop degranulation responses.

β-Hexosaminidase activity assay

RBL-2H3 cells were dispensed into 96-well plates at a concentration of 2×10^5 cells/well and incubated over-night at 37 °C in 5% CO₂. Cells were sensitised with anti-DNP IgE for 2 h. Cells were washed twice with Siraganian buffer before adding the test sample to each well and incubated for 1 h, followed by challenge with DNP-albumin, antigen for 1 h. The

reaction was stopped by cooling in an ice bath for 10 min. The supernatant (50 µL) was transferred into 96-well microplate and incubated with 50 µL of substrate solution for 1 h. The reaction was stopped by adding a stop solution. The absorbance was measured with a microplate reader at 405 nm.

Contents of TGF-β in serum

TGF-β contents were measured in accordance with a previous study (Oka et al. 2020). Briefly, whole blood was left undisturbed for 30 min at room temperature and subsequently centrifuged at 10,000 rpm at 4 °C for 10 min. Supernatants were collected as blood serum. Serum samples were stored at –80 °C until analysis. Serum TGF-β levels were measured using an ELISA (Promega, Madison, WI) in accordance with the manufacturer's recommended protocol.

RNA isolation and quantitative Real-Time PCR of TGF-β mRNA

Total RNA was extracted from Caco-2 cells using Sepasol RNA I super (Nacalai Tesque). cDNA synthesis was performed using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster

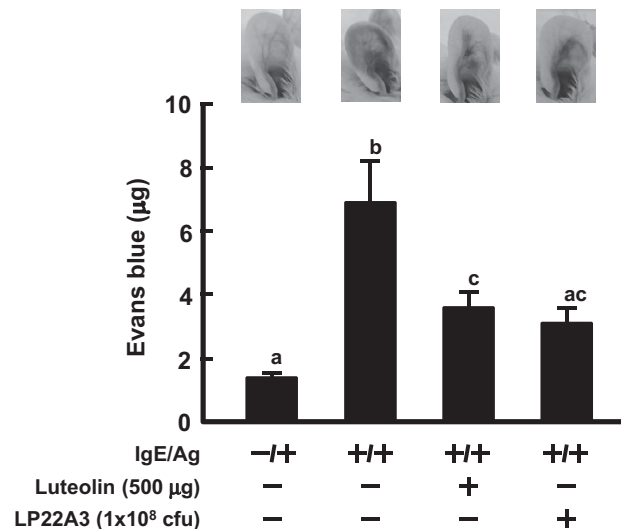


Figure 1. Anti-allergic activity of LP22A3 PCA reaction. LP22A3 (1×10^8 cfu/mouse/day) and luteolin (500 µg/mouse/day used as a positive control) were orally administered to mice for 10 days before anti-DNP IgE sensitisation. After 24 h, DNP-albumin and Evans blue were injected via tail vein as an antigen challenge. Evans blue stained ears were photographed. The ear-absorbed dye was extracted with formamide, and absorbance at 620 nm was measured. Values represent means $\pm$ SD ($n = 4$). The animal experiments were conducted with two independencies. Different superscript letters after values indicate statistical significance between groups ($p < 0.05$).

City, CA) in accordance with the manufacturer's protocol. Quantitative PCR assays were analysed using Applied Biosystem 7500 Fast Real-Time PCR system with TaqMan gene expression assay. TaqMan gene expression assay was purchased from Applied Biosystems; β -actin (Mm00607939_s1) and mouse TGF- β (Mm01178820_m1).

Statistical analysis

All the data were presented as mean $\pm$ standard deviation. Statistical significance among each group were evaluated by analysis of variance (ANOVA) and Tukey–Kramer test to determine differences between groups. Statistical significance was defined as $p < 0.05$.

Results

Oral administration of LP22A3 suppressed anaphylaxis in PCA model mouse

Oral administration of LP22A3 significantly inhibited the IgE-mediated PCA reaction (Figure 1). Weight and drinking amounts of LP22A3-fed mice show no significant difference compared with the control group throughout the experiment (data not shown). Moreover, oral administration of LP22A3 for 10 days significantly increased TGF- β level in blood to be approximately 2.7 fold (Figure 2). It was assumed that the anti-allergic activity of LP22A3 might be associated with TGF- β secretion in blood. To confirm this

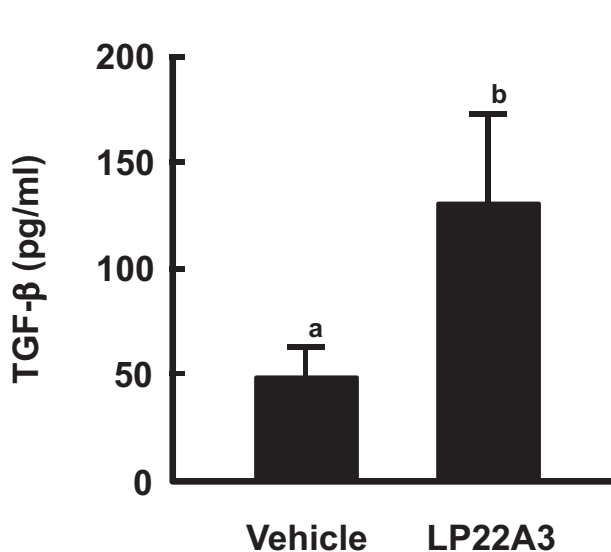


Figure 2. Upregulation of TGF- β secretion in blood by oral administration of LP22A3. TGF- β level in serum prepared from mice in Figure 1 was measured by ELISA. Values represent means $\pm$ SD ($n = 4$). Different superscript letters after values indicate statistical significance between groups ($p < 0.05$).

hypothesis, mice were injected intraperitoneally with recombinant TGF- β (17.5 ng/mouse/day) every day for 10 days. It was reported in our previous study that the exogenous TGF- β injection increased TGF- β contents in the blood to be more than 200 pg/mL which is almost equal to TGF- β levels by the oral administration of LP22A3 in this experiment (Oka et al. 2020). The increase of TGF- β contents in blood by exogenous TGF- β injection decreased Evans blue release into mice ears to the same level as LP22A3 treatment, indicating that IgE-mediated PCA reaction was inhibited (Figure 3).

Suppression of degranulation of RBL-2H3 cells by LP22A3 involves an interaction with Caco-2

To investigate whether LP22A3 affected mast cell directly, LP22A3 was applied to IgE-sensitised RBL-2H3 cells. As shown in Figure 4(A), LP22A3 did not show suppression of β -hexosaminidase from RBL-2H3, but luteolin did. However, LP22A3 could suppress β -hexosaminidase release from RBL-2H3 at the same levels of luteolin when LP22A3 was applied in the apical side of Caco-2/RBL-2H3 co-culture system (Figure 4(B)). These results suggested that LP22A3 affects RBL-2H3 cells through Caco-2 cells, indicating that the involvement of intestinal epithelial cells is an important factor in the anti-allergic activity of LP22A3.

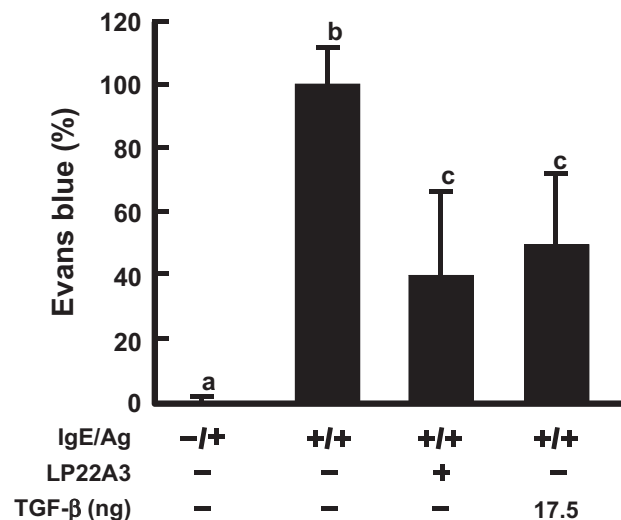


Figure 3. Influence of exogenous TGF- β on PCA reaction. TGF- β (17.5 ng/mouse/day) were intraperitoneally injected to mice for 10 days. Values represent means $\pm$ SD ($n = 4$). The animal experiments were conducted with two independences. Different superscript letters after values indicate statistical significance between groups ($p < 0.05$).

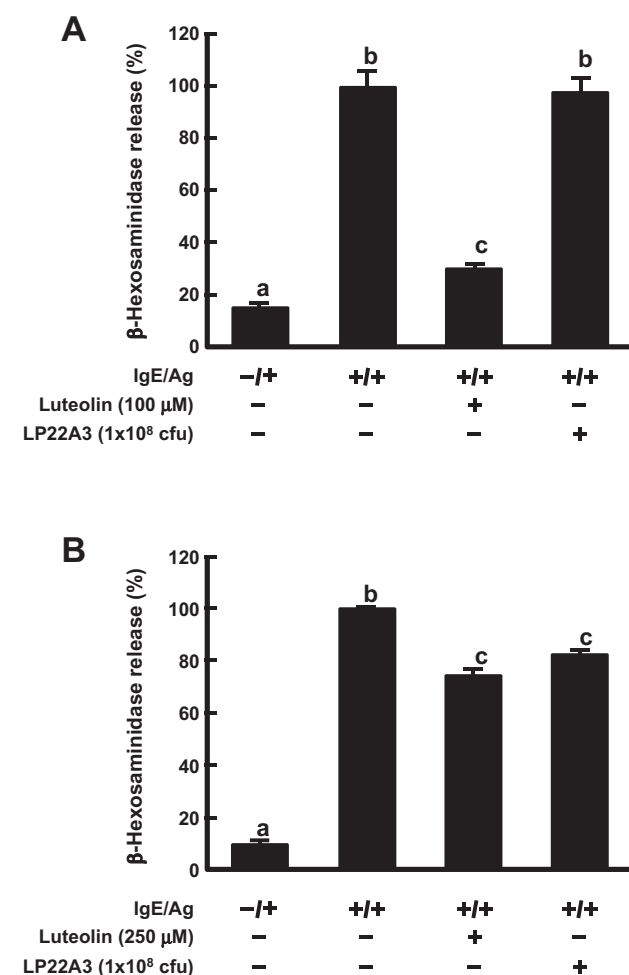


Figure 4. Effect of LP22A3 treatment on degranulation of RBL-2H3 cells in monoculture and co-culture system of Caco-2/RBL-2H3 cells. LP22A3 (1×10^8 cfu/mL) was applied directly to RBL-2H3 cells (A) or into apical side of Caco-2/RBL-2H3 cells (B) prior to sensitise anti DNP-IgE antibody. Degranulation of RBL-2H3 cells was evoked by DNP-albumin. Values represent means $\pm$ SD ($n = 3$). Different superscript letters after values indicate statistical significance between groups ($p < 0.05$).

Anti-allergic activity of LP22A3 depends on TGF- β production

In the PCA reaction, it was ascertained that TGF- β contents in blood were increased (Figure 2). To confirm the involvement of TGF- β secretion through intestinal epithelial cells, RBL-2H3 cells were pre-treated with an anti-TGF- β antibody for 30 min in the basolateral side before adding LP22A3 into the apical side of the co-culture system. LP22A3 stimulation from the apical side increased TGF- β mRNA expression (Figure 5(A)). Moreover, pre-treatments of anti-TGF- β antibody or irrelevant IgG show no effects in the expression of TGF- β mRNA which remained almost the same level as when stimulated with LP22A3. However, pre-treatment with anti-TGF- β

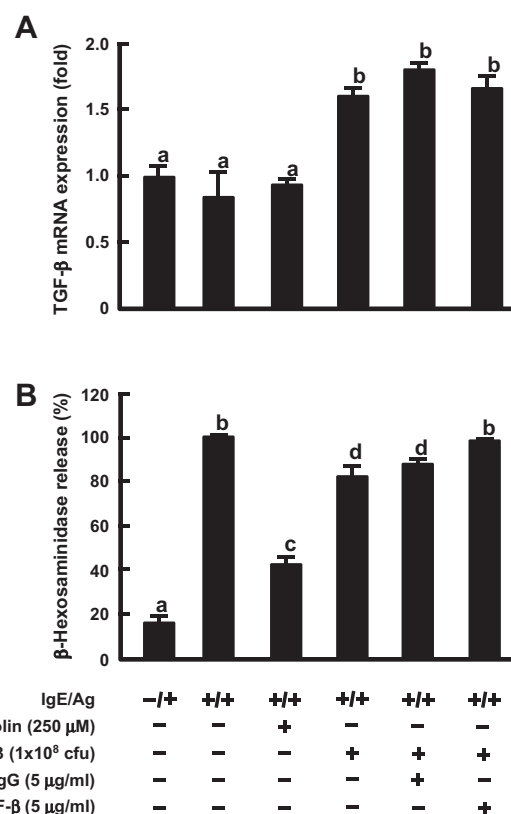


Figure 5. Effect of anti-TGF- β antibody on β -hexosaminidase release from RBL-2H3 cells in co-culture system. Anti-TGF- β antibody was applied to the basolateral side of the co-culture system prior to LP22A3 treatment. (A) TGF- β mRNA expression from Caco-2 cells was measured by qPCR. (B) β -Hexosaminidase release was measured by β -hexosaminidase assay. Values represent the means $\pm$ SD ($n = 3$). Different superscript letters after values indicate statistical significance between groups ($p < 0.05$).

antibody in the basolateral side eliminated the suppression of β -hexosaminidase release although the pre-treatment of irrelevant IgG did not (Figure 5(B)). Thus, TGF- β secretion from Caco-2 cells stimulated with LP22A3 plays an inhibitory role of β -hexosaminidase release from RBL-2H3.

Discussion

Many clinical trials revealed that several *Lactobacillus* strains, including *L. rhamnosus* GG, *L. acidophilus* L-92, and *L. casei* Shirota, were effective in the prevention of early atopic disease in children and of allergic symptoms in patients sensitive to Japanese cedar pollen (Kalliomäki et al. 2001; Ishida et al. 2005; Tamura et al. 2007). Therefore, a lot of studies mainly focussed on the therapeutic and preventive effects of LAB in allergic diseases.

In this study, the inhibitory effect of LP22A3 on type I allergy was evaluated by the PCA model as

in vivo and co-culture model composed of human intestinal epithelial Caco-2 cells and rat basophilic leukaemia RBL-2H3 cells as *in vitro* experiments. It was demonstrated that LP22A3 possessed anti-allergic activity and induced to the secretion of TGF- β in blood. TGF- β is a multifunctional cytokine that plays pivotal roles in diverse biological processes, including the regulation of cell growth and survival, cell and tissue differentiation, development, inflammation, immunity, haematopoiesis, and tissue remodelling and repair (Lee et al. 2006; Letterio and Roberts 1998). TGF- β can decrease Fc ϵ RI expression, and inhibits mast cell degranulation (Gomez et al. 2005). Intraperitoneal injection of exogenous TGF- β (17.5 ng/day/mouse) for 10 days to mice demonstrated the inhibition of Evans blue exudation. This TGF- β concentration has been reported to increase TGF- β levels in the blood to be more than 200 pg/mL which is almost equal to its concentration of mice administered with LP22A3 (Oka et al. 2020).

It was ascertained that the increase of TGF- β secretion in blood by LP22A3 administration was found to contribute to the allergy suppression in PCA reaction. The co-culture system composed of Caco-2 and RBL-2H3 cells have already been proven to be effective in investigating intestinal-mediated anti-allergic effects (Yamashita et al. 2016). LP22A3 treated in the apical side indicated the suppression of β -hexosaminidase release from RBL-2H3, but its direct treatment to RBL-2H3 did not. These results indicated that intestinal cells are involved to exert the influence against RBL-2H3 cells in β -hexosaminidase activity by LP22A3. The pre-treatment of anti-TGF- β antibody and irrelevant IgG indicated no influence of TGF- β mRNA in Caco-2 cells compared to LA22A3 stimulation, indicating that TGF- β secretion was comparable in each group. On the other hand, it was demonstrated that TGF- β neutralising antibody could cancel the inhibition of β -hexosaminidase activity by LP22A3 treatment. Collectively, these outcomes indicated that LP22A3 exerted its inhibitory activity through its unique ability to promote TGF- β production from Caco-2 cells. Torii et al. (2007) reported that oral administration of *Lactobacillus acidophilus* Strain L-92 increased TGF- β production from cells Peyer's patches.

Conclusions

In conclusion, this paper demonstrates that the oral administration of LP22A3 possesses anti-allergic activity in PCA reaction and that its activity is mediated

by TGF- β production through intestinal epithelial cells using a co-culture system of Caco-2/RBL-2H3 cells. However, we were unable to determine the mechanism of how LP22A3 induced TGF- β production in the blood through intestinal epithelial cells. Clarification of the relationship between anti-allergic activity and TGF- β production by LP22A3 may provide a clue of why certain LABs exert anti-allergic activity.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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