Double-culture Method Enhances the in Vitro Inhibition of Atopy-inducing Factors by Lactococcus lactis

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We analyzed whether lactic acid bacteria could control the expression of IL-4 and IL-13 in activated mast cells and whether these bacteria could inhibit the activity of transcription factors such as GATA-1, GATA-2, NF-AT1, NF-AT2, and NF-κB p65. We previously described a technique for identification of lactic acid bacteria with anti-atopy functionality by confirming increased expression of CD4+/CD25+/Foxp3+ in T cells. We also confirmed that a double-culture method increased the anti-bacterial activity of these lactic acid bacteria against Staphylococcus aureus (S. aureus). In the present study, we characterized the effect of lactic acid bacteria cultured by this double-culture method on inhibition of allergic inflammatory reactions of RBL-2H3 mast cells, a cellular model of atopic dermatitis. The strongest anti-allergic effects of the lactic acid bacteria were seen in the following order: Lactococcus lactis broth cultured with medium containing Lactobacillus plantarum culture supernatant > Lc. lactis > Lc. lactis broth cultured with medium containing Lb. plantarum culture supernatant > Lb. plantarum. Thus, Lc. lactis cultured in medium containing Lb. plantarum culture supernatant had the strongest inhibitory effect on the differentiation of mast cells during allergic reactions, which may be mediated through the selective regulation of expression of relevant genes.

Key words: Anti-allergy, anti-atopy, lactic acid bacteria, Lactobacillus plantarum, Lactococcus lactis

Introduction

Immunity can be largely categorized as innate immunity and adaptive immunity, and atopic dermatitis is associated with abnormal innate and adaptive immune responses. In chronic lesions of atopic dermatitis, IFN-γ, IL-5, IL-12, and GM-CSF are up-regulated, and Th1 inflammatory cytokines such as IL-12 and IL-18 play an important role in maintaining chronic state. In contrast, Th2 inflammatory cytokines such as IL-4 and IL-13 are critical to acute lesions of atopic dermatitis. In general, atopic dermatitis is caused by an imbalance of Th1/Th2 reactions and is worsened by increase of IgE due to the suppression of Th1 cells by Th2 cytokines [26, 27]. When the activated mast cells are exposed to the allergen again, it contributes to chronic allergic reactions through the increased transcription of cytokine genes such as IL-4 and IL-13 [31]. Therefore, if the expression of IL-4 and IL-13 could be controlled, it may be possible to alleviate the allergic reaction or suppress the progression of the acute allergic reaction to the chronic state. It is known that the expression of Th2 cytokines is specifically controlled by transcription factors such as NF-AT, NF-κB p65, and GATA which are selectively expressed in mast cells [16, 44]. The anti-allergic effect of lactic acid bacteria, especially on atopic dermatitis, was demonstrated in animal model and cell culture system [14]. Additionally, a previous report showed that babies from mothers who ingested Lactobacillus GG before and after delivery had significantly reduced onset of atopic dermatitis up to 4 years old [19]. Although probiotics is used to treat some diseases, poor understanding of the mechanism of action and strain specific functionality limits the general use of them.

The improvement of living environment and medication are applied for alleviating allergic symptoms, however, there is no prominent and qualified treatment without adverse effect and with prolonged efficacy yet. Many clinical studies evaluated the effect of lactic acid bacteria on immunological diseases such as allergy and atopic dermatitis. Some studies reported that intake of fermentation products from Lactobacillus rhamnosus, Bifidobacterium lactis, Lb. acidophilus, and Lb. casei had strain-specific therapeutic effects on allergy...
Various studies also showed that probiotics, killed lactic acid bacteria, and lactic acid bacteria lysates induced anti-cancer effect [29], improved absorption of lactose and milk proteins [15], lower serum cholesterol levels [42], treatment for rheumatoid arthritis [5], and intestinal regulation [20].

In our previous report [18], we screened functional lactic acid bacteria having immuno-stimulating activity and anti-allergic effect by using FACS analysis and developed media composition for optimal culture condition. We also tested antimicrobial activity of lactic acid bacteria against *Staphylococcus aureus*. Based on the increase of CD4+/CD25+/foxp3+ T cell subpopulation, the immuno-stimulating effect by using FACS analysis and developed media composition for optimal culture condition. We also tested antimicrobial activity of lactic acid bacteria against *Staphylococcus aureus*. Based on the increase of CD4+/CD25+/foxp3+ T cell subpopulation, the immuno-stimulating activity and anti-cancer effect [29], improved absorption of lactose and milk proteins [15], lower serum cholesterol levels [42], treatment for rheumatoid arthritis [5], and intestinal regulation [20].

**Materials and Methods**

**Cell culture**

RBL-2H3 cells, a rat mast cell line, were obtained from the Korea Cell Line Bank (Seoul, Korea). These cells were grown in minimal essential medium (MEM, Gibco-BRL, NY, USA) supplemented with 10% fetal bovine serum (FBS, Gibco-BRL, NY, USA). The media contained penicillin (100 U/ml, Gibco-BRL, NY, USA) and streptomycin (100 µg/ml, Gibco-BRL, NY, USA). Cells were grown at 37°C in a humidified 5% CO2 incubator. RBL-2H3 cells were seeded in a 100 mm culture dish and then cultured for 24 hr. A nontreated group was used as a normal group, and a group treated with 50 ng/ml of phorbol 12-myristate 13-acetate (PMA) only and 0.5 µM of ionomycin was used as a PI-control group. After culturing, the positive control group (PL-cyclosporin A(CsA)) was treated with 10 µg/ml CsA, and the experimental group (PL-LAB) was treated with 10 µg/ml lactic acid bacteria homogenate. Cyclosporin A is a strong immunosuppressive agent against the T-cell immunity by inhibiting the secretion of IL-1 and IL-2 and by suppressing the proliferation of T helper cell [11, 38]. After 1 hr incubation they were stimulated with 50 ng/ml of PMA and 0.5 µM of ionomycin, and washed with cool PBS 6 hr later. The cells were then collected using a scraper and the supernatant was removed by centrifuge.

**The culture method for lactic acid bacteria**

MRS broth supplemented with 10% of either culture medium or supernatant of *Lb. plantarum* was autoclaved. The *Lc. lactis* was inoculated at a content of 1% into the pressured-sterilized broths and was standing-cultured at 35°C in an incubator for 24 hr to obtain Lb.p-sup.+Lc. lactis and Lb.p-cult.+Lc. lactis. To prepare the medium containing *Lb. plantarum* supernatants, the *Lb. plantarum* cultured medium was centrifuged at 10,000 rpm for 10 min and then added.

**Preparation of lactic acid bacteria**

The lactic acid bacteria cultured in MRS broth for 24 hr was centrifuged at 9,000 rpm for 10 min, and the collected lactic acid bacteria were washed twice not to be influenced by the low pH generated by the lactic acid. The lactic acid bacteria were then completely broken down by sonication five times for 20 min (output control : 2, duty cycle (%) : 30, and 20 kHz) using an ultrasonic processor (Branson Ultrasoundics Model 450, Fisher, MA, USA). Insoluble materials were then removed by centrifuging at 9,000 rpm for 10 min. The collected supernatant was filtered with a 0.45-µm syringe filter to obtain a cytoplasmic fraction.
Western blot

Cells were treated with a hypotonic buffer using a nuclear extract kit (Abcam, Cambridge, MA, UK) on ice for 15 min and then centrifuged to collect the cytoplasmic fraction. The nuclear remaining in the tube were treated with complete lysis buffer on ice for 30 sec and then centrifuged to obtain the nuclear fraction.

The obtained protein was quantified by BCA protein quantitative analysis. Antibodies for Western blot are anti-NF-AT1, anti-NF-AT2, anti-NF-κB p65, anti-GATA-1, anti-GATA-2, and lamin B antibodies (Thermo Fisher Scientific, MA, USA). The band densities were calculated using Image J software and then used to calculate the inhibition of atopic dermatitis.

Real-time PCR

Total RNA was extracted using Trizol reagent (Invitrogen, CA, USA). The RNA was added to cDNA synthesis cocktails (deoxynucleotide triphosphate (dNTP), TaKaRa, Tokyo, Japan; moloney murine leukemia virus reverse transcriptase Promega, WI, USA; RNase inhibitor, Promega, WI, USA; RNAzolB, Tel-Test, FL, USA=1:1:1). Primers for IL-4, IL-13, and β-actin were diluted to the proper concentration and added. SYBR Green (SYBR master mix, Applied Biosystems, CA, USA) and cDNA were mixed and real-time PCR (Quantitative Real-Time PCR, Applied Biosystems, CA, USA) was performed under cycling conditions of 95℃ for 3 min, followed by 40 cycles of 95℃ for 15 sec, 60℃ for 15 sec, and 72℃ for 20 sec (Table 1) [12].

Statistical analysis

The experimental groups were statistically analyzed by unpaired Student’s t-test in statistical analysis software. Significance was defined as $p<0.05$.

Results

Inhibition of NF-AT transcription factors

Expression of IL-4 and IL-13 is controlled by various transcription factors such as NF-AT1, NF-AT2, GATA-1 and GATA-2 [12, 22]. The transcription factor NF-AT is present in a non-activated, i.e., phosphorylated, state in the cytoplasm, and its dephosphorylation is caused by activation of calcineurin, which increases the concentration of Ca$^{2+}$ in response to stimulation of an antigen receptor. Dephosphorylated NF-AT is transferred into the nucleus and bound to an antigen receptor response element present in the IL-2 promoter in activated $T$ cells to induce the expression of various cytokines (e.g., IL-3, IL-4, IL-5, GM-CSF, TNF-α, and IFN-γ) and cell surface molecules (e.g., CD40L, FasL, and IL-2Ra) which are essential for inducing an immune reaction in im-

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Table 1. Primer sequences for real-time PCR analysis

| Target gene | Primer | Sequence |
|-------------|--------|----------|
| IL-4        | Forward| 5'-GGATGTAACGACAGCCCTCT-3' |
|             | Reverse| 5'-GTGTTCCCTTGTCGCGTAAG-3' |
| IL-13       | Forward| 5'-CAGTTGCAATGCCATCCACA-3' |
|             | Reverse| 5'-AGCCACATCGAGGCCCTTT-3' |
| β-actin     | Forward| 5'-AGCCAGTGTGATGATCGCAAG-3' |
|             | Reverse| 5'-AACAGTCGCCCTAGAAGCAT-3' |
munocytes [9, 45]. However, excessive activation of NF-AT causes transplantation rejection, autoimmune diseases, and cardiovascular disorders, so it is necessary to suppress the immune reaction to treat such diseases. The effect of lactic acid bacteria on the expression of NF-AT1 and NF-AT2 in mast cells was analysed by western blot. After treatment with homogenate of the lactic acid bacteria, the expression of NF-AT1 protein in the experimental group stimulated with PI showed the greatest inhibition in the Lc. lactis group (63.1%) and the inhibition of the expression of NF-AT2 protein was greatest in the Lb. p-sup. + Lc. lactis group (68.8%). It is likely that the allergic inflammatory reaction was controlled by inhibition of NF-AT1 and NF-AT2 expression (Fig. 1).

**Inhibition of NF-κB p65 transcription factor**

It is known that a κB binding motif is present in the 5’ of most inflammation-mediating cytokine genes whose expression could be controlled by the NF-κB transcription factor. Thus, the NF-κB pathway is a converging pathway for the expression of inflammation-mediating cytokines [3, 42]. NF-κB is a complex of proteins consisting of p50 and p65 heterodimers and is maintained at a non-activated state by binding with an inhibitor of NF-κB. When this complex is dissociated by external stimulation, NF-κB moves into the nucleus and binds to a κB element (GGGAATTC) in a promoter region of specific genes, thereby inducing the transcription of the genes. Representative inflammation-mediating factors whose transcription is activated through this pathway include TNF-α, IL-2, IL-6, IL-8, lymphotoxin, GM-CSF, β-IFN, and adhesion molecules [2, 6, 8, 10]. To determine the effect of the homogenate of lactic acid bacteria, the expression of NF-κB p65 proteins was measured by western blot analysis. The result displayed that the expression of NF-κB p65 proteins in the experimental group stimulated with PI after treatment with Lb. p-sup. + Lc. lactis was largely down-regulated as compared with the control group (30.0%). Thus, lactic acid bacteria suppresses allergic inflammatory reactions by controlling the signal transduction mechanism of NF-κB (Fig. 2).

**Inhibition of GATA transcription factors**

It is important to systematically express genes for correctly mature cells. GATA transcription factor groups are species-specific transcription factors that play such a role in controlling the expression of GATA-1 and GATA-2 genes. GATA transcription factors have DNA-binding regions and well-maintained zinc finger regions, and these factors cause transcriptional activation by binding to a (A/T)-GATA(A/G) sequence. Their expression is limited to specific tissues and cells; for example, GATA-1 is expressed in red blood cells, megakaryocytes, and mast cells [16, 32, 35]. GATA plays an important role in the expression of IL-13 in T cells as well as the expression of IL-4 and IL-5. In addition, GATA

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Fig. 1. Effect of Lc. lactis cultured with medium containing Lb. plantarum culture supernatants on NF-AT signaling in RBL-2H3 cells induced with PI. Abbreviations: Lb. p-cult + Lc. lactis, Lc. lactis culture broth with a medium containing Lb. plantarum culture broth; Lb. p-sup + Lc. lactis, Lc. lactis culture broth with a medium containing Lb. plantarum supernatants. RBL-2H3 cells were pretreated with cyclosporin A or lactic acid bacteria extract for 1 hr, and then stimulated with PMA-ionomycin for 6 hr. After lysis buffer was added, one part of the lysate was subjected to Western blotting with NF-AT protein. Another part of the lysate was directly subjected to SDS-PAGE and immunoblotting with the indicated antibodies. NF-AT proteins were selected as positive controls for anti-NF-AT1 and anti-NF-AT2 respectively.

Fig. 2. Effect of Lc. lactis cultured with medium containing Lb. plantarum culture supernatants on NF-κB p65 signaling in RBL-2H3 cells induced with PI. Abbreviations were the same as Fig. 1. RBL-2H3 cells were pretreated with cyclosporin A or lactic acid bacteria extract for 1 hr, and then stimulated with PMA-ionomycin for 6 hr. After lysis buffer was added, one part of the lysate was subjected to Western blotting with NF-κB p65 protein. Another part of the lysate was directly subjected to SDS-PAGE and immunoblotting with the indicated antibodies. NF-κB p65 proteins were selected as positive controls for anti-NF-κB p65.
promotes the transcriptional activation of IL-13 gene in combination with the activation of STAT6 and NF-κB [33, 37]. To confirm the effect of lactic acid bacteria on the expression of GATA-1 and GATA-2 in mast cells, GATA-1 and GATA-2 proteins were measured by western blot analysis. The result showed that the expression of GATA-1 proteins was suppressed in the Lc. lactis group stimulated with PI after treatment with lactic acid bacteria (89.0%), and that the expression of GATA-2 proteins was largely suppressed in the Lb. p-sup. + Lc. lactis group (66.5%). Therefore, lactic acid bacteria suppresses allergic inflammatory reactions by inhibiting the expression of the transcription factors GATA-1 and GATA-2, which blocks the signal transduction mechanism of GATA (Fig. 3).

**Inhibition of IL-4 cytokine production**

Th2 cytokines are over-expressed in the acute skin lesions of atopic dermatitis. Th2 cytokines induce allergic reactions and also suppress innate immune reactions. In addition, IL-4 generated by the Th2 immune response inhibits recovery after the damage of skin wall, the synthesis of ceramides, and the expression of peptide antibiotics. In addition, IL-4 has been reported to inhibit the expression of desmoglein 3, loricrin, and involucrin, thereby directly deteriorating the function of the skin wall [1, 13, 21, 23]. Hypersensitive immunity is basically caused by the production of IgE in response to allergens. IL-4 is essential to produce IgE in allergic reaction. Because IL-4 is produced by T helper 2 cells, Th2 immune reactions play an important role in inducing hypersensitive immunity in response to exogenous antigens. IL-4 expression is controlled through NF-κB and NF-AT transcription factors [46]. After RBL-2H3 cells were stimulated with PI after treatment with the lactic acid bacteria homogenate and CsA, their expression of IL-4 mRNA was measured by real-time RT-PCR. The expression of IL-4 mRNA was decreased by 86.4% in the Lc. lactis 10 μg/ml group. As measured by ELISA, IL-4 production was significantly reduced by 91.5% in the Lc. lactis 10 μg/ml group compared with the control group. Thus, the Lc. lactis may effectively inhibit allergic inflammatory reactions by down-regulating the production of IL-4 in mast cells. Similar results were shown in the Lc. lactis culture with medium containing Lb. plantarum supernatant (Fig. 4 and 5).

**Inhibition of IL-13 cytokine production**

IL-13 is correlated with the physiological changes induced by allergic inflammation in many tissues. The mechanism
underlying the control of IL-13 expression in mast cells remains unclear, however, IL-13 expression is known to be controlled by many transcription factors including NF-AT and GATA [30]. NF-AT2 plays an important role in controlling IL-13 transcription along with GATA. GATA-2 promotes IL-13 production in mast cells [22, 28]. IL-13 mRNA expression in RBL-2H3 cells treated with CsA and the homogenate of lactic acid bacteria after stimulation with PI was analyzed by real-time PCR, which confirmed that IL-13 mRNA expression was significantly decreased by 64.8% in the *Lb. p*-sup. + *Lc. lactis* 10 µg/ml group. IL-13 protein production was significantly diminished by 94.9% in the *Lb. p*-sup. + *Lc. lactis* 10 µg/ml group compared to the control group. Therefore, the *Lc. lactis* culture with medium containing *Lb. plantarum* supernatant could effectively suppress allergic inflammatory reactions by significantly inhibiting the production of IL-13 in mast cells. Similar results were shown in the *Lc. Lactis* (Fig. 6 and 7).

Discussion

To alleviate allergy symptoms, the living environment could be improved or drugs may be used, but the effect is temporary or side effects may occur. Thus, there are no truly effective treatment methods yet. Recently, effective treatment methods having few side effects and a high immune effect have been studied. Clinical studies have found that
the effectiveness of therapies for immune disease such as atopic dermatitis could be improved through the ingestion of lactic acid bacteria, which protects from the outbreak of allergies [34, 39, 43]. It has reported that strain-specific allergies can be cured by eating fermentation products of lactic acid bacteria, such as Lactobacillus rhamnosus, Bifidobacterium lactis, Lactobacillus acidophilus, and Lactobacillus casei [40]. In addition, many studies reported that probiotics, and homogenates of lactic acid bacteria are effective for preventing cancer [29], improving absorption of lactose and milk proteins [15], decreasing cholesterol [42], treating rheumatoid arthritis [5], and enhancing intestinal regulation [20].

Thus, in terms of the functionality of lactic acid bacteria, this study confirmed that antimicrobial and anti-allergic activity was increased by culturing the lactic acid bacteria in a medium containing the supernatant of lactic acid bacteria. We concluded that lactic acid bacteria can effectively suppress allergic inflammatory reactions by inhibiting activity and differentiation of mast cells and by selectively controlling their relevant gene expressions. Based on the protein expression of transcription factors such as NF-AT, NF-κB p65, and GATA, and the expression of IL-4 and IL-13, it is suggested that the lactic acid bacteria homogenate inhibits allergic inflammatory reactions in the order of Lb. p-sup. + Lc. lactis group, Lc. lactis, Lb. p-cult. + Lc. lactis group, and Lb. plantarum by inhibiting the productions of IL-4 and IL-13 in activated mast cells. In particular, the expression of IL-4 and IL-13 mRNA is likely reduced through the blockade of GATA and NF-AT signal transduction, which is mediated by the inhibition of GATA-1, GATA-2, NF-AT1, and NF-AT2 NF-κB p65 transcription factors. This effect is similar to that observed in antimicrobial activity (anti-S. aureus) in a previous report [18]. Accordingly, it can be considered that when Lc. Lactis is cultured in a medium containing Lb. plantarum supernatant, it could repress the activity and differentiation of mast cells by regulating related gene expression.

Table 2 shows a summary of anti-atopic effects of lactic acid bacteria based on the culture method in this study (lactic acid bacteria sample 10 μg/ml). In the future, we are going to evaluate the anti-atopic effect from this experiment in animal models.

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유산균이 활성화된 비만세포에서 발현하는 IL-4와 IL-13을 조절할 수 있는지 분석하였고, GATA-1, GATA-2, NF-AT1, NF-AT2, NF-κB p65 전사인자의 활성을 억제하는지를 실험적으로 규명하였다. 이전 연구에서 T cell에서 CD4+/CD25+/foxp3+ 증가를 실험하여 항아토피 기능성이 있는 유산균을 탐색하였고, Staphylococcus aureus에 대한 항균력을 증가시키는 유산균의 이중배양법을 확인하였다. 여기서는 RBL-2H3 비만세포를 이용하여 이 배양법으로 배양한 유산균이 아토피 피부염의 원인이 되는 allergy염증반응에서 얼마나 억제능을 갖는지 알아보았다. 그 결과 Lc. lactis culture with medium containing Lb. plantarum supernatants > Lc. lactis > Lc. lactis culture broth with medium containing Lb. plantarum culture broth > Lb. plantarum의 순으로 나타났다. 이 cell 수준(level of mast cells)에서의 순서는 이전 연구의 level of microorganisms (anti-S. aureus)에서의 아토피 유발인자 억제능 순서와 같다. 따라서 세포수준에서도 Lb. plantarum 배양상층액을 첨가한 배지에 Lc. lactis 배양한 경우가 활성화된 비만세포의 allergy 반응으로의 분화 및 활성을 가장 잘 억제하고 관련 유전자 발현을 선택적으로 조절하는 anti-allergy 효과를 나타내다고 사료된다.