論文の内容の要旨

応用生命化学　専攻
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論文題目 Study on the Mechanisms of Immunomodulators Using an Atopic Dermatitis Mouse Model
(アトピー性皮膚炎モデルを用いた免疫調節物質の作用機構に関する研究)

Atopic dermatitis (AD) is a kind of disease in which itchy eczema repeatedly improves and deteriorates chronically. The pathophysiology of AD is skin barrier dysfunction, allergic inflammation, and pruritus, but the detailed mechanism is not fully understood. Studies in immunology and allergology have pointed out an imbalance in the ratio of Th1/Th2 type cells in atopic dermatitis, and Th2 cells become dominant as the symptoms progress. There is also an increase in the total immunoglobulin E (IgE) in the patient's serum.

Various mouse models have been developed in order to elucidate the mechanism of atopic dermatitis, develop improvement methods, and search for substances which have improving effect. In this study, I used a 2,4-dinitrochlorobenzene (DNCB) induced atopic dermatitis mouse model to examine the improving effects of several immunomodulators. The immunomodulators I investigated in this study are GcMAF, ferulic acid (FA) anserine and *Lactococcus lactis* subsp. *cremoris* YRC3780.

GcMAF is vitamin D3-binding protein-derived macrophage activating factor. According to the results of clinical trials, it showed the effect to improve skin symptoms in atopic patients. FA is an organic compound that exists as a phytochemical on the cell wall of plants. Anserine is an imidazole dipeptide found in meat such as pork beef and chicken. *L. cremoris* YRC3780 strain is a lactic acid bacterium derived from kefir. FA, anserine and *L. cremoris* YRC3780 have been reported or found in our laboratory to suppress Th2 responses and/or enhance Th1 responses.

Chapter 1 Preparation of GcMAF from human serum

In this chapter, I purified Gc protein from human serum and produced GcMAF from Gc protein. Since Gc protein can bind to vitamin D_3, a 25-hydroxy vitamin D_3 (25-OH-D_3)-immobilized affinity column was first prepared according to a previous report. 25-hydroxy Vitamin D_3 3,3'-aminopropyl ether was dissolved in dimethylformamide (DMF), and the solution pH was adjusted by adding K_2CO_3, then incubated with TOYOPEARL AF-Epoxy-650 M resin in ambient nitrogen at 50 °C for 4 h. Excess ligands on 25-OH-D_3-immobilized resin were removed by washing with DMF and ultrapure water. The epoxy group of the resin was blocked with 1 M ethanolamine at room temperature overnight. After washing with ultrapure water, the resin was placed in a polypropylene column. Then, for the first-time purification, equal amounts of human serum and column buffer were mixed, passed through the affinity column. The column was washed with NaCl-containing column buffer, eluted with guanidine hydrochloride. Eluents were collected and dialyzed in column buffer. Since impurities in each of the recovered fractions of eluent were observed on SDS electrophoresis images, further purification was performed using an ion exchange column. The fraction from ion exchange column in

which the band was observed was collected and concentrated to obtain Gc protein. Subsequently, sialidase, mannosidase and β-galactosidase enzymes were immobilized with NHS-activated Sepharose 4 fast flow. The purified Gc protein was treated by immobilized enzyme to produce GcMAF.

The prepared Gc protein and GcMAF were reduced-alkylated, trypsin-digested, finally desalted, and analyzed by LC-MS/MS. The results of LC-MS/MS analysis revealed that the prepared protein was Gc protein. For the prepared GcMAF analysis, results showed there were fragments which sugar chains were still bound, but there were also many fragments of GcMAF which belong to Gc1S type. Based on this result, for the next experiments, enzyme treatment time of Gc protein was lengthened.

Chapter 2 Effect of immunomodulators on DNCB induced atopic dermatitis mouse model

In this chapter, I built a DNCB induced BALB/c mouse model and injected or fed mice with GcMAF, FA, anserine and *L. cremoris* to detect whether these immunomodulators can alleviate the symptoms of AD in this mouse model.

To build the mouse model, back fur of 6-week BALB/c mice were removed with an electronic razor and mice were divided into 3 groups (control group, back challenge group, ear challenge group). 2% DNCB was applied to the back of mice for twice sensitization in the first week. After that, a back challenge of 0.2% DNCB in the same way as sensitization or ear challenge with 0.3% DNCB by pipette were performed once every two days for a total of three weeks. Serum IgE levels were measured by ELISA and gene expression level of cytokines related to AD of back skin and ears, and draining lymph nodes (DLN) near ears (for ear challenge group) were measured by RT-PCR. I found that after two weeks of challenge, IgE levels increased significantly in the ear-challenged and back-challenged groups. In the ear challenge group, expression levels of various cytokines related to AD were significantly increased, whereas the back-challenge group did not show much change. Based on these results, I decided that the challenge would be performed in the ear for 2 weeks in the next administration experiment.

Then, using the ear challenge DNCB mouse model, mice were injected with GcMAF or gavaged with FA, anserine and *L. cremoris* for 2 weeks. However, the results showed that IgE levels increased significantly both in DNCB group and GcMAF group compared with the control group, but no difference was observed between these 2 groups. Moreover, the gene expression levels of the cytokines were changed in DNCB group compared with the control group, but no change was observed between DNCB group and GcMAF group. Ear thickness also increased significantly in the DNCB group mice as the times of challenges increased compared to the control group, but DNCB and GcMAF group maintained comparable. In FA, anserine and *L. cremoris* gavaged group, ear thickness of mice showed a significant decrease compared with DNCB group. However, although IgE level in FA group tended to be decreased compared with DNCB group, I could not find any changes in gene expression of AD related cytokines in DLN whole cells or in mesenteric lymph nodes (MLN), Peyer's patches (PP) $CD4^+$ T cells. For anserine and *L cremoris* group, although there was no significant change in the gene expression levels of $CD4^+$ T cells cytokines in MLN and PP, administration of *L. cremoris* resulted in an increasing trend of IFN-γ gene expression in ear tissue compared to DNCB group. The IFN-γ expression levels of DLN also tended to upregulate in the anserine group.

As described above, GcMAF was prepared and administered to a mouse model of atopic dermatitis in BALB/c mice induced by DNCB. However, no improvement of symptoms was observed. On the other hand, oral administration of FA, anserine, and *L. cremoris* alleviated the symptoms of mice ears. Although there were no significant changes in

total IgE levels or gene expression of cytokines, administration of *L. cremoris* YRC3780 and anserine tended to increase IFN-γ expression.

Chapter 3 Mechanisms of Anserine and *L.cremoris* Alleviates DNCB induced atopic dermatitis in different phases

Improvement of AD symptoms on ear by oral administration of anserine and *L. cremoris* can be observed in AD mouse model after 1-week sensitization and 2 weeks challenge, but the detailed mechanisms still need to be clarified. So, in this chapter, I did some in vitro experiments to try to explain the mechanisms how anserine and *L. cremoris* affected immune cells. Moreover, since pathogenic process of atopic dermatitis has the different phases, based on my AD mouse model, I tried different challenge and gavage duration and tried to find the effect of these 2 immunomodulators on different phases of AD mouse model.

In RAG23-3/BALB/c mice in vitro whole spleen cells experiments, I found IL-4 production in the supernatant was significantly decreased by adding *L. cremoris*. Moreover, low levels of *L. cremoris* also enhanced the production of IFN-γ and IL-12 (p40/p70), tendency of IFN-γ was the same as IL-12 (p40/p70). However, no obvious tendency was observed by adding anserine. Then, I narrowed the range of cells try to find detailed effect of anserine and *L. cremoris*. So, I added anserine or *L. cremoris* in co-cultured antigen presenting cells and $CD4^+$ T cells system and analyzed cytokine levels in supernatant. The results of this experiment showed the reduced content of IL-4 in a dose dependent manner by adding anserine, but obvious change in IFN-γ and IL-12 (p40/p70) could not be identified. The same as whole spleen cells experiment, low levels of *L. cremoris* had the same tendency of increasing IFN-γ and IL-12 (p40/p70), and the decrease effect of IL-4 was also observed. Next, I focused on the effect of anserine and *L. cremoris* on co-cultured intestinal DCs and $CD4^+$ T cells. In MLN-DCs and $CD4^+$ T cells co-culture experiment, the results showed both anserine and *L. cremoris* enhanced the gene expression of IFN-γ and IL-12 p40. Since IL-12 p40 is secreted by DCs and can induce the production of Th1 cytokine IFN-γ, these results confirmed the regulation of Th1 response of anserine and *L. cremoris* in MLN-DCs and $CD4^+$ T cells co-culture system. Moreover, in PP-DCs and $CD4^+$ T cells co-culture system, a significant reduction of IL-4 gene expression was found by anserine and *L. cremoris* addition. I also examined the direct effect of anserine and *L. cremoris* on differentiated Th1 and Th2 cells. However, anserine addition did not show any effect on Th1 and Th2 cells. For *L. cremoris* addition, IFN-γ production was reduced in Th1 differentiated cells and IL-4 production also decreased in Th2 differentiated cells. Although I analyzed $CD4^+Foxp3^+$ regulatory T cells by flow cytometry to investigate the effect of anserine and *L. cremoris*, obvious enhancement could not be found in this experiment. The results suggested that the effect of anserine may be restricted to certain antigen-presenting cell populations such as intestinal dendritic cells, while *L. cremoris* may act on various types of cells to produce IL-12 and IFN-γ and suppress IL-4.

As indicated in chapter 2, I also found upregulation effects of IFN-γ of anserine and *L. cremoris* in AD mice which were challenged 2 weeks. Then, I lengthened the challenge time and gavage times to nearly 3 weeks (23 days). Although the alleviation of AD symptoms which is reflected by ear thickness can be observed in both anserine and *L. cremoris* group compared to AD group with the increase of challenge treatment, total serum IgE levels after whole experiment was not reduced in either 2 immunomodulator groups. However, the enhancement of IL-4 production in DNCB group and reduction of IL-4 production in *L. cremoris* group in splenocytes of mice indicated that a systematic

Th2 response was elicited in DNCB group and *L. cremoris* may systematically regulate this response. Moreover, a slightly increased gene expression of Foxp3 in MLN $CD4^+$ T cells and increase in frequency of $CD4^+Foxp3^+$ T cells were observed in *L. cremoris* group. Next, the early phase of AD mice, which were challenged and gavaged for 1.5 weeks (11 days), were also investigated. This time, a significant decrease of serum total IgE levels in anserine group and *L. cremoris* group compared with DNCB group were observed. Moreover, increased gene expression of IL-4 in ear tissue of DNCB group was observed which was decreased in both the anserine group and *L. cremoris* group. However, obvious tendency of IL-4 production in splenocytes could not be detected. The down regulation of IL-4 gene expression also occurred in PP $CD4^+$ T cells in both 2 immunomodulator groups corresponding to the in vitro PP-DCs and $CD4^+$ T cells co-culture system experiment. Generation in MLN $Foxp3^+$ $CD4^+$ T cells could not be detected. These results indicated that in the early AD phase, Th2 response mainly occurred in ear tissue of mice, anserine and *L. cremoris* can down-regulate the production of IL-4 in ear probably by the suppression of IL-4 derived from PP $CD4^+$ T cells. Because of the decrease of IL-4 in ear tissue and PP $CD4^+$ T cells, serum IgE also tended to be decreased. In middle phase, the immunomodulatory effect of anserine and *L. cremoris* was the enhancement of IFN-γ locally, and they had no effect on serum IgE. In late AD phase, Th2 response became systemic and *L. cremoris* regulated this response by down regulating IL-4 from PP and produce $CD4^+Foxp3^+$ regulatory T cells in MLN.

Conclusion

Taken together, this study highlights the effect of anserine and *L. cremoris* on treating DNCB induced atopic dermatitis by moderating Th1/Th2 balance, decreasing serum IgE and enhancing the generation of Tregs in the intestinal immune system by acting on certain immune cells in different phases of AD.

Published paper

Morita, Y., **Wang, R**., Li, X., Muramatsu, T., Ueda, M., Hachimura, S, et al. "Improved preparation of group-specific component (Gc) protein to derive macrophage activating factor." Protein Expression and Purification 175 (2020): 105714.