論 文 の 内 容 の 要 旨

応用生命化学専攻
平成 20 年度博士課程　　　　入学
氏　　名　　　王　璽凱
指導教員名　　　清水　誠

論文題目
Promotive effect of dietary phytochemicals on induction of regulatory T cells
（植物由来食品因子による制御性 T 細胞の分化誘導促進効果に関する研究）

Introduction

Allergy is defined as an inappropriate, overactive and adverse immune reaction to exogenous harmless antigens including those derived from food. Although various therapies have been developed, using anti-allergic dietary factors to prevent allergies would be a promising strategy. Flavonoids are the most common polyphenolic compounds and the anti-allergic functions of dietary flavonoids have been reported. Aryl hydrocarbon receptor (AhR) is a transcription factor that plays an important physiological role in toxicological system-mediated regulation. Recent evidence also suggests that oral administration of AhR-ligands activates AhR and induces regulatory T cells (Tregs). Tregs are important in immunological tolerance, which is associated with food allergy and oral tolerance. Taken together, considering that some flavonoids are ligands of AhR, AhR-agonistic flavonoids must be potent to modulate the immunological function via Tregs induction, and therefore, to regulate different types of allergies including foodborne allergies. The aim of this study is (i) to screen potential AhR-agonistic dietary phytochemicals for use as Treg-inducible dietary factors, (ii) to characterize Tregs induced by dietary factors and to investigate their

immune-regulatory activities, and (iii) to evaluate *in vivo* effect of Treg-inducible dietary factors administered perorally.

Chapter 1. Screening and evaluation of phytochemicals on the induction of Tregs *in vitro*

In this chapter, we attempted to find new anti-allergic dietary factors by screening dietary phytochemicals with AhR-agonistic activity in order to identify compounds inducing functional Tregs *in vitro*. NIH3T3 cells transfected with a xenobiotic responsive element (XRE)-luciferase reporter plasmid were used to monitor the effect of dietary phytochemicals on AhR-dependent transcriptional activity. Among 25 phytochemical samples tested, apigenin, (+)-catechin, fisetin, genistein, hesperetin, naringenin, quercetin hydrate, and rutin showed markedly high luciferase activities, and we found that only naringenin possesses the activity of inducing Tregs *in vitro* via a TGF-β-independent pathway. This result indicates that not all AhR ligands can induce Tregs and suggests that the specific structure of naringenin may contribute to its immune-regulatory activity.

To further confirm whether naringenin affects the induction of Tregs via AhR-ligand interaction, we next investigated the effect of AhR antagonists—resveratrol, TMF, and CH-223191—on the induction of Tregs. We found that the immunosuppressive effect induced by naringenin was attenuated upon the co-treatment with AhR antagonists. These AhR antagonists are reported to have different characteristics and action mechanisms one another, suggesting that the immunosuppressive activity of naringenin is regulated by its direct interaction with the ligand-binding site of the AhR molecule. Taken together, naringenin was shown to have the immue-regulatory activity on *in vitro* Tregs induction via an AhR-dependent pathway.

Chapter 2. Characterization and investigation of the function of Tregs induced by naringenin

In Chapter 2, we investigated the characteristics and immunomodulatory function of the Tregs induced by naringenin *in vitro*. We found that (i) the populations of both $CD4^+Foxp3^+$ T cells and $CD4^+CD25^+Foxp3^+$ T cells were dose-dependently increased in $CD4^+$ T cells activated and differentiated *in vitro* in the presence of naringenin, and that (ii) $CD4^+Foxp3^+$ T cells induced by naringenin expressed higher levels of CTLA4, GITR, LAP, and FR4 compared to the control. These results suggest that naringenin induced $CD4^+CD25^+Foxp3^+$ Tregs which express typical Treg-associated surface molecules. We found that Foxp3 mRNA transcript levels were dose-dependently up-regulated by the induction of naringenin. NCABS2 is the functional non-evolutionarily conserved AhR

binding site in the *Foxp3* promoter and might participate in controlling Foxp3 expression. Naringenin exhibited higher NCABS2-driven luciferase activity by the reporter gene expression assay. Interestingly, AhR mRNA expression was also up-regulated in $CD4^+$ T cells activated in the presence of naringenin in a naringenin-dose dependent manner.

We next investigated the expression of immune-suppressive cytokines by the naringenin-induced Tregs. Our data demonstrated that $CD4^+$ T cells induced by naringenin at relatively low concentrations (6–12.5 μM) secreted significantly higher amount of IL-10 than the control $CD4^+$ T cells and the $CD4^+$ T cells induced by relatively high concentrations (25–50 μM) of naringenin. Moreover, we found that anti-IL-10 and anti-TGF-β antibodies attenuated the suppressive function of Treg cells induced by naringenin at low and high concentrations, respectively. We further determined that the suppressive effect of T cells induced by naringenin at high concentrations was mediated by membrane-bound TGF-β in a cell-cell contact mechanism.

Taken together, it was suggested that naringenin induced IL-10 producing Tr1-like cells and $Foxp3^+$ Treg cells depending on the doses. We assume that naringenin up-regulated the AhR expression in $CD4^+$ T cells dose-dependently and naringenin-activated AhR might bind Foxp3 promoter to regulate the induction of $CD4^+Foxp3^+$ Tregs expressing typical Treg-associated markers. In addition, in a specific range of concentration, naringenin induced IL-10 producing T cells via an AhR-dependent and/or unknown mechanisms.

Chapter 3. Evaluation of the immune-regulatory effect *in vivo* by oral administration of naringenin

AhR-ligands have been reported to induce Tregs *in vivo* that control and regulate the immunological tolerance, and we have found in this study that naringenin was a potent AhR agonistic dietary flavonoid and induced Tregs *in vitro*. Hence, we attempted to investigate whether naringenin possesses the immune-regulatory effect *in vivo* in terms of the induction of Tregs in BALB/c mice and DO11.10 mice. First we found that the populations of both $CD4^+Foxp3^+$ T cells and $CD4^+CD25^+Foxp3^+$ T cells in spleen (SPL) and mesenteric lymph node (MLN) were up-regulated by feeding BALB/c mice with naringenin. The percentages of T cells expressing Treg-associated molecular markers in whole $CD4^+$ SPL, $CD4^+$ MLN, and $CD4^+$ Peryer's patch (PP) T cells were mostly significantly up-regulated by naringenin administration. Furthermore, IL-2 production by $CD4^+$ SPL T cells stimulated with

anti-CD3 and anti-CD28 antibodies *in vitro* was significantly down-regulated by naringenin administration, suggesting that the $CD4^{+}Foxp3^{+}$ T cells, which do not secrete IL-2, included in the $CD4^{+}$ SPL T cells exerted the suppressive effect against other normal $CD4^{+}$ T cells unaffected by naringenin administration. These results suggested that oral administration of naringenin to BALB/c mice is potent to expand $CD4^{+}Foxp3^{+}$ Tregs *in vivo*. The percentages of $CD4^{+}IL\text{-}10^{+}$ T cells and $CD4^{+}TGF\text{-}\beta^{+}$ T cells among PP $CD4^{+}$ T cells were also increased by oral administration of naringenin.

The up-regulation activity on expanding $CD4^{+}Foxp3^{+}$ Tregs of oral naringenin administration suggested that naringenin might enhance the induction of oral tolerance. Hence, we subsequently examined the immune-regulation effect of oral naringenin administration in the oral tolerance induction model using DO11.10 mice, which express rearranged T-cell receptor gene specific to 323-339 residues of ovalbumin and have been used for the investigation of T-cell development and immune-regulation. Although the generation of OVA-specific $Foxp3^{+}$ Tregs was observed after oral administration of OVA, oral administration of naringenin showed no enhancing effect on OVA-specific $CD4^{+}Foxp3^{+}$ Tregs. In contrast, IL-10 production by SPL and PP $CD4^{+}$ T cells from DO11.10 mice fed naringenin was significantly higher than by those from the control mice. However, no promoting effect of naringenin administration on oral tolerance induction was observed in the conditions applied in this study.

In our *in vitro* experiments, naringenin directly affected $CD4^{+}$ T cells which were stimulated with anti-CD3 and anti-CD28 antibodies. However, in the case of oral administration, naringenin might also affect other immune cells, such as dendritic cells playing an important role in T cell activation. Therefore, further investigations on the effects of naringenin on other immune cells and its mechanisms of action are required to clarify the immune-regulatory effect of naringenin *in vivo*.

In conclusion, both the *in vitro* and *in vivo* results demonstrated that naringenin is potent to induce $CD4^{+}Foxp3^{+}$ Tregs and to up-regulate IL-10 producing $CD4^{+}$ T cells in some conditions. These promising results, we believe, would provide us a new approach for developing anti-allergy food via the potential immunomodulatory activity of naringenin, and have wide range potential applications such as health supplements, quasi drug, as well as functional foods.