論文の内容の要旨

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論文題目

Effect of apple-derived condensed tannins and pomegranate-derived polyphenols on cytokines responses of intestinal epithelial cells

（リンゴ由来縮合タンニンとザクロ由来ポリフェノールが腸管上皮細胞のサイトカイン応答に与える影響に関する研究）

Introduction

Polyphenols, one of the most common groups of plant secondary metabolites, have received considerable interest over the past few years due to their presumed role in the prevention of various degenerative diseases. Apple (*Malus spp., Rosaceae*) are a widely consumed, rich source of polyphenols. Apple components investigation showed that apple peel contains an abundance of procyanidins and their polymers (apple condensed tannins; ACT). Accumulated evidence indicates that the consumption of apple and apple products could reduce risks of cardiovascular diseases, cancers, asthma, diabetes, obesity, and pulmonary dysfunction. These pharmacological activities from apple and apple processed products have been shown to be associated with ACT, which abound in unripe apple peel more than ripe one.

Pomegranate (*Punica granatum L.*) fruit is widely consumed fresh and in processed forms as juice, jam, and wine. Pomegranate peels and leaves were used in the traditional medicine of different Asian cultures for the treatment of a variety of ailments. Phytochemical studies have revealed that ellagitannins (ETs) and anthocyanins (ANs) represent the most abundant polyphenols in pomegranate aril and peel. Hydrolysable tannin, stictinin is the main phenolic component present in pomegranate leaves. Importantly, numerous scientific researches demonstrate that the health benefits of pomegranate and their products have been positively associated with the polyphenols in these fruits.

It is well-known that cytokines play a key role in the intestinal immune system. Inflammatory bowel diseases (IBD) are chronic inflammatory diseases of the gastrointestinal tract. Inflammatory cytokine interleukin 6 (IL-6) and chemokine CXCL10 have been identified as important cytokines in IBD. IL-6 is a pleiotropic cytokine that exerts its proinflammatory effects largely by means of its soluble IL-6 receptor. It has been reported

that altered IL-6 production has been found in IBD affected patients. Chemokines CXCL9, CXCL10, and CXCL11 are known to activate a common receptor CXCR3, which is commonly expressed on activated T cells, B cells, and NK cells. These three chemokines recruit activated T cells into the intestinal mucosa. In addition, high levels of CXCL10 release has also been found in patients with IBDs. On the other hand, these cytokines are important in activation of the immune system to prevent infection in the intestine at non inflammatory steady state.

Recently, numerous scientific studies suggested that polyphenols appear as promising candidates for up-regulating or down-regulating cytokines expression. However, the influences of apple peel-derived polyphenols and pomegranate-derived polyphenols on IL-6 and CXCL10 expression in intestinal epithelial cells have not yet been revealed. The present study aimed to investigate the effects of ACT and pomegranate aril extract, peel extract, and leaf extract on the IL-6 and CXCL10 expression in intestinal epithelial cells, and to examine the effects of apple extracts and pomegranate extracts on cell signaling pathways to uncover the mechanism.

Chapter 1. Polymerization degree-dependent inhibitory effect of apple condensed tannins on IL-6 production in mouse intestinal epithelial cells

Poly I:C is structurally similar to dsRNA as a respective agonist for toll-like receptor 3 (TLR3) which selectively triggers TLR3-dependent up-regulation of IL-6 and CXCL10 production. This chapter describes the effects of ACT on Poly I:C-induced IL-6 expression in a mouse small intestinal epithelial cell line, MoS13 cells. Since ACT is a complex flavonoid polymer, consist of oligomeric (-)-epicatechin of 2-14 mers, the effect of smaller fractions of ACT (2-mer) and larger fractions of ACT (7-mer) on IL-6 expression was also investigated.

ACT at 25, 50 μg/mL was found to inhibit Poly I:C-mediated IL-6 mRNA expression, transcription and protein expression by MoS13 cells. In addition, I evaluated the effect of ACT on signaling pathways by western blot analysis. This effect was found to be regulated by p38, MEK1/2, JNK-MAPKs, and NF-κB/IκB pathways. However, the smaller fraction of ACT (monomer and dimer) had no effect on the release of IL-6 from Poly I:C-induced MoS13 cells. Whereas Poly I:C-mediated IL-6 production and mRNA expression by the cells was robustly inhibited by larger fractions of ACT (7-mer). These results suggested that higher concentration of ACT and larger fractions of ACT down-regulated Poly I:C-induced IL-6 expression polymerization-degree dependently by suppressing NF-κB, MEK1/2, and JNK activity.

Chapter 2. Apple polyphenols regulate chemokines CXCL9, CXCL10, and CXCL11 expression in intestinal epithelial cells via activation of MAPKs pathway

In Chapter 2, I investigated the effect of ACT on Poly I:C-induced expression of chemokines CXCL9, CXCL10 and CXCL11 by the intestinal epithelial cells. MoS13 cells were treated with ACT at four concentrations (0.7, 3.1, 12.5, 50 μg/mL) in the presence of Poly I:C. To my surprise, the data demonstrated that ACT stimuli at 3.1 or 12.5 μg/mL significantly up-regulated Poly I:C-induced CXCL9, CXCL10, and CXCL11 expression. Interestingly, ACT (12.5μg/mL) did not up-regulate Pam3CSK3 (TLR1/2 ligand)- and LPS (TLR4 ligand)-induced expression of these chemokines. In addition, ELISA and luciferase reporter gene assay analysis revealed that ACT (12.5 μg/mL) significantly increased the production of CXCL10 and CXCL11 in Poly I:C-stimulated MoS13 cells and increased promoter activity of the CXCL10 gene as well. Furthermore, the analysis using inhibitors of signaling pathways suggested that suppression of PKC, MEK1/2, JNK, p38, and PI3K pathways significantly prevented the up-regulation of CXCL10 gene expression. Subsequently, the effect of ACT on Poly I:C-induced signaling pathway was evaluated by western blot analysis. It was confirmed that ACT enhanced the phosphorylation of MEK, JNK, and p38, and the nuclear translocation of IRF3, whereas ACT stimuli did not increase the phosphorylation of IκB. These results suggest that ACT up-regulates PI3K-IRF3 and PKC-MAPKs (MEK1/2, JNK, p38) signaling pathways. In *in vivo* experiments, an increased CXCL10 expression was observed in the intestine of BALB/c mice receiving ACT by oral administration.

The results shown in Chapter 1 and Chapter 2 demonstrated that ACT at different concentrations showed different regulating effects on IL-6 and CXCL10 expression. On the transcriptional level, it has been known that CXCL10 gene promoter activation is regulated by NF-κB and IRF3 as well as AP-1 working downstream of MAPKs signaling pathways, whereas IL-6 gene promoter was regulated by NF-κB and AP-1 but not by IRF3. The fact that ACT up-regulated a PI3K-IRF3 signaling pathway, which was obvious when ACT (12.5 μg/mL) was added, as well as a MAPK pathway, and also down-regulated a NF-κB/IκB pathway, which was clearly shown at ACT (50 μg/mL), seems to explain the up-regulation of CXCL10 expression and the down-regulation of IL-6 expression by ACT in Poly I:C-stimulated MoS13 cells.

Chapter 3. Pomegranate peel-derived polyphenols stimulate CXCL10 gene expression and production via activation of PKC-MAPKs and PI3K-IRF3 signaling pathways in intestinal epithelial cells

The aim of the study in Chapter 3 was to investigate the effects of pomegranate aril extract (PAE), pomegranate peels extract (PPE), pomegranate leaves extracts (PLE) on chemokines expression in the intestine *in vivo* and *in vitro*. BALB/c mice were fed a normal diet and either water or water containing 1% PAE, 1% PPE, and 1% PLE *ad libitum* over a period of two weeks. Interestingly, only oral administration of 1% PPE significantly up-

regulated CXCL9 and CXCL10 gene expression in the intestinal mucosa. In addition, we examined the enhancement effect of PPE on the chemokine gene expression *in vitro* using MoS13 cells. I found that treatment of MoS13 cells with PPE at a concentration of 50 μg/ml for 20 h, significantly increased CXCL9, CXCL10, and CXCL11 gene expression. It was observed that PPE stimulation significantly enhanced the production of CXCL10 and the transcriptional activation of CXCL10 gene as well.

To reveal the mechanism, first, we evaluated the effect of each inhibitor on respective signaling pathways. We observed that the induction of CXCL10 gene expression was suppressed by pharmacological inhibitors of PKC (RO31-8220), PI3K (LY294002), MEK1/2-ERK (PD98059, U0126), P38 (SB203580), or JNK (SP600125), suggesting the involvement of PKC and MAPKs pathways in CXCL10 gene expression. In contrast, NF-κB inhibitor did not suppress PPE-induced CXCL10 gene expression. Importantly, the western blot analysis results suggested that PPE stimulation significantly increased the phosphorylation of MEK1/2, ERK1/2, JNK, but did not affect the phosphorylation of p38 and IκB. Furthermore, we observed that PPE treatment significantly increased the nuclear translocation of IRF3, while did not affect the nuclear translocation of NF-κ κ B. In conclusion, these results suggest that PPE enhanced CXCL10 expression in intestinal mucosa via activation of PKC, MEK1/2, ERK1/2, and JNK signaling pathways and a PI3K-IRF3 pathway.

Conclusions

In this whole works, I have investigated the effect of ACT on IL-6 and CXCL10 gene expression, and also compared the effect of PAE, PPE, and PLE on CXCL10 gene expression in mouse intestinal epithelial cells. It was observed that ACT showed different regulating effect on IL-6 and CXCL10 gene expression in Poly I:C-induced MoS13 cells. In Chapter 1 ACT (50 μg/mL) inhibited IL-6 expression predominantly via suppressing NF-κB/IκB and PKC-MAPKs (MEK1/2, JNK, p38) signaling pathways. In contrast, in Chapter 2, I first found that ACT (12.5 μg/mL) up-regulated Poly I:C-induced CXCL10 expression predominantly via activation of PI3K-IRF3 and PKC-MAPKs (MEK1/2, JNK, p38) signaling pathways, but not via an NF-κB/IκB signaling pathway. In Chapter 3, PPE was found to up-regulate CXCL10 expression predominantly via activation of PI3K-IRF3 signaling pathway and PKC-MAPKs (MEK1/2, ERK1/2, and JNK) signaling pathway without inducing NF-κB/IκB signaling pathway. These findings suggest that apple peel extract and pomegranate peel extract may regulate cytokine and chemokine expression in intestinal epithelial cells via different signaling pathways. These results also support that consumption of apples and pomegranate may help protect against intestine disorder by decreasing pro-inflammatory cytokines and enhancing gut immunity with increasing chemokines in the normal steady state.