学 位 論 文 の 要 旨

氏名　　清水 保彦

学 位 論 文 名　Effects of the Japanese Traditional Medicine Bu-zhong-yi-qi-tang on Th2 Responses via Up-regulation of Toll-like Receptor 4

発 表 雑 誌 名　Asian Rhinology Journal
（巻, 初頁～終頁, 年）　(**1**: 42-51 2013)

著 者 名　Yasuhiko Shimizu, Noriaki Aoi, Ichiro Morikura, Takafumi Fuchiwaki, Hideyuki Kawauchi

論 文 内 容 の 要 旨

INTRODUCTION

Allergic rhinitis is an inflammatory disease associated with a Th2 response, airway infiltration by eosinophils, and nasal hyper-reactivity. Th1 cells, into which naive clusters of differentiation(CD)4^+ T cells preferentially differentiate in the presence of IL-12, IL-15, IL-18 and interferon (IFN)-γ secrete IL-2, IFN-γ and tumor necrosis factor (TNF)-α not only for induction of cell-mediated immunity but also for down-regulation of Th2 responses. Therefore, cytokines involved in Th1-biased response are thought to regulate Th2-mediated allergic response. Many kinds of traditional Japanese herbal medicines have immunomodulating activities, e.g., B cell mitogenic activity, activation of macrophages, enhancement of natural killer (NK) activity and action on hematopoietic stem cells. Bu-zhong-yi-qi-tang (Japanese name: Hochu-ekki-to, HOT) is composed of 10 species of medicinal plants and is used for treatment of symptoms such as weakness caused by fatigue and weakness after illness. HOT has been recently reported to upregulate Toll-like receptor (TLR)-4 expression on monocytes. TLRs have been shown to play important roles in the recognition of bacterial components. Ten members of the TLR family have been reported so far. Among various TLRs, TLR-4 mediates lipopolysaccalide (LPS) signal transduction in collaboration with other molecules, such as CD14, MD-2, myeloid differentiation factor 88 (MyD88), and Toll IL-1R domain containing adapter protein (TIRAP)/MyD88-adapter-like(Mal). To this end, we have successfully examined the inhibitory effect of HOT on Th2 responses by an enhancement of IL-12 production from macrophages via up-regulation of TLR-4 expression.

MATERIALS AND METHODS

Female C3H/HeN and HeJ (TLR4-gene mutant) mice were used at 7–9 weeks of age. Raw264.7, a murine macrophage cell line, and HEK293 cells were maintained in RPMI medium with 10% fetal calf serum. Adherent cells from peritoneal exudates of naïve C3H/HeN or C3H/HeJ mice were used as a source of murine macrophages. Spray-dried HOT was prepared as a hot water extract from 10 species of medicinal plants : Ginseng radix, Atractylodis rhizoma, Astragali radix, Angelicae radix, Zizyphi fructus, Bupleuri radix, Glycyrrhizae radix, Zingiberis rhizoma, Cimicifugae rhizome and Aurantii nobilis pericarpium. Concentrations of IL-4, IL-12 p40 and IFN-γ in the culture supernatants were measured by commercial ELISA kits. For western blotting, anti-murine monoclonal IL-5 and IL-13 antibodies were purchased from Genzyme. Mice were each intraperitoneally immunized with 100μg of Ovalbumin(OVA) absorbed on 1 mg of Alum on days 0 and 7. This was followed by intranasal (i.n.) challenge with 20 μl of 1% OVA solution from day 21 to day 28. The mice were orally administered with 1000 mg/kg of HOT (suspended in phosphate-buffered saline, PBS) or PBS alone on days 1–8. Spleen cells were prepared 24 hours after the last inhalation. Total cellular RNA from Raw 264.7 cells or murine peritoneal macrophages was extracted with RNAzol B. Levels of OVA-specific IgE, IgG_1 and IgG_{2a} were determined by ELISA. Spleen cells were incubated on a nylon wool column at 37°C in 5% CO_2 for 60 min. T cells (5×10^5) and Mitomycin C (MMC)-treated naive splenocytes (5×10^5) were cultured in 96-well cell culture plates with 200 μg OVA. The cultured supernatants were collected and the amounts of secreted IL-4 and IFN-γ in the supernatants were determined by ELISA. After the i.n. challenge with OVA or PBS, the mice were placed in the observation cage again and the numbers of sneezes were counted for 5 minutes. Coronal nasal sections were then stained with hematoxylin and eosin, and the number of eosinophils in each side of the posterior edge of the nasal septum was counted microscopically.

Mice were maintained according to the guidelines for animal treatment at the research center of Shimane university and this protocol was approved by animal and use committee in Shimane university.

RESULTS AND DISCUSSION

First of all, we investigated the effect of HOT on TLR4 gene expression. HOT alone did not influence the TLR4-specific mRNA expression in Raw264.7 cells. LPS, a ligand of TLR4, also had no effect alone on the TLR4-specific mRNA expression. However, in our present study, the treatment with HOT before stimulation with LPS was proven to result in a significant increase of TLR4-specific mRNA expression in macrophages. Likewise, HOT did not directly influence IL-12 production by macrophages *in vitro*, although pretreatment with HOT enhanced the IL-12 production of macrophages stimulated with LPS. Furthermore, interestingly, peritoneal macrophages derived from C3H/HeN mice,orally administered with HOT, produced a large

amount of IL-12, when those were stimulated with LPS *in vitro*. However, no influence on IL-12 production was found in peritoneal macrophages derived from C3H/HeJ mice, TLR4-deficient natural mutant mice. These results suggest that HOT cooperates with LPS to upregulate TLR4-specific mRNA expression and IL-12 production of macrophages. To be next, we assessed the *in vivo* effects of HOT on a murine allergic rhinitis model. Oral treatment with HOT resulted in inhibition of OVA-specific IgE and IgG_1 production in C3H/HeN mice. Besides, OVA-specific IL-4 production by splenic T cells, derived from HOT-treated C3H/HeN mice, was significantly decreased in comparison with those of control mice. In contrast, there was no difference in levels of OVA-specific serum Igs or cytokine production of splenic T cells, between C3H/HeJ mice treated with HOT and those untreated with HOT. After the intranasal challenge of OVA, counts of sneezing, esoinophilic infiltration and IL-5 expression in nasal mucosa were significantly decreased in HOT-treated C3H/HeN mice. On the other hand, no significant differences were seen between C3H/HeJ mice treated with HOT and those untreated with HOT. These findings indicate that HOT has an inhibitory effect in a murine allergic rhinitis model, by enhancing IL-12 production from macrophages via TLR4 signaling pathway. We demonstrated that HOT cooperates with LPS to up-regulate TLR4 gene expression on macrophages *in vitro* and that pretreatment with HOT enhanced IL-12 production by macrophages with LPS stimulation and inhibited Th2 responses *in vivo*. We found that oral administration of HOT in the induction phase preferentially induced Th1 cells producing IFN-γ. One of the mechanisms for induction of Th1 response by HOT administration is the up-regulation of TLR4-gene expression in macrophages, as shown in our study. Macrophage/dendritic cell-derived cytokines such as IL-12, IL-15 and IL-18 might be partially responsible for early IFN-γ production from NK and NKT cells and consequently Th1 cell differentiation. Alternatively, HOT may contain ligands for NK and NKT cells and directly stimulate the production of IFN-γ. Thus, the results of the present study suggest that methods to enhance IFN-γ production might be clinically useful in the prophylaxis of allergic rhinitis.

CONCLUSION

Oral administration of HOT reduced Th2 responses in the induction phase of allergic responses via up-regulation of TLR4-gene expression. Our results therefore might offer a novel approach using HOT for the treatment of allergic disorders, such as allergic rhinitis.