学 位 論 文 の 要 旨

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学 位 論 文 名　Japanese Traditional Medicine, Senn-kinn-naidaku-sann Up-regulates Toll-like Receptor 4 and Reduces Murine Allergic Rhinitis

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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 $CD4^+$T cells preferentially differentiate in the presence of IL-12, IL-15, IL-18 and IFN-γ secrete IL-2, interferon (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. Senn-kinn-naidaku-sann (SKNS) is composed of 9 species of medicinal plants and is used for treatment of symptoms such as general fatigue due to weakness and infection. Bu-zhong-yi-qi-tang was reported to up-regulate Toll-like receptor (TLR)4 expression on monocytes. TLR4 mediates LPS signal transduction, which is considered to be effective for infection, may influence TLR4 expression and IL-12 production in macrophages. In this study, we examined the effects of SKNS on a murine allergic rhinitis model by enhancement of IL-12 production from macrophages via up-regulation of TLR4 expression. We used C3H/HeN mice and C3H/HeJ mine lacking TLR4 signaling pathway to investigate the

effect of SKNS on TLR4.

MATERIALS AND METHODS

Female C3H/HeN and HeJ mice were used at 7–9 weeks of age. A mouse macrophage cell line, RAW264.7, was maintained in RPMI with 10% FCS. Adherent cells from peritoneal exudates of naive C3H/HeN or C3H/HeJ mice were used as mouse macrophages. Spray-dried SKNS was prepared as a hot water extracted from 9 species of medicinal plants, including Ginseng Radix 3g, Angelicae Radix 3g, Astragali Radix 3g, Cnidii Rhizoma 2g, Sinomeni Caulis et Rhizoma 2g, Platycodi Radix 2g, Magnoliae Cortex 2g, Angelicae Dahuricae Radix 1g and Glycyrrhizae Radix 1g. Concentrations of IL-4, IL-12 p40 and IFN-γ in the culture supernatants were measured by using commercial ELISA kits. Anti-murine monoclonal IL-5 and IL-13 antibodies for use in Western blotting assay were purchased from Genzyme. Total cellular RNA from Raw264.7, HEK293 and mouse peritoneal macrophages was extracted with RNAzol B. For western blot analysis, proteins were obtained from the nasal mucosa of each mouse 12 hours after the final nasal challenge followed by detection using an enhanced chemiluminescence system. Mice were each intraperitoneally immunized with 100μg OVA absorbed on 100 μl of Alum on days 0 and 7. This was followed by daily intranasal (i.n.) challenge with 25 μg of OVA diluted by sterile normal saline from day 21 to day 28. In the SKNS treatment group, mice were orally administered with SKNS (suspended in phosphate-buffered saline (PBS)) or PBS everyday on days 1–7 using gastric tubes. Spleen cells were prepared 24 h after the last inhalation. Levels of OVA-specific IgE, IgG1 and IgG2a 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 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 min. Coronal nasal sections were then stained with hematoxylin and eosin.

All animals 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, we assessed the effects of SKNS on the expression of TLR4 on murine macrophages. SKNS alone enhanced the expression of TLR4 mRNA. In vitro, though pretreatment with SKNS enhanced IL-12 production by macrophages following stimulation with LPS. Peritoneal

macrophages derived from mice that had been orally administered SKNS produced a large amount of IL-12 following stimulation with LPS in vitro, although no effect on IL-12 production by peritoneal macrophages derived from C3H/HeJ mice, TLR4-gene mutant mice, was observed. SKNS is therefore thought to affect the expression of TLR4 mRNA on macrophages and enhance IL-12 production by macrophages stimulated with LPS.

Next, we assessed the effects of SKNS on a murine allergic rhinitis model. Oral treatment with SKNS successfully resulted in inhibition of OVA-specific IgE and IgG1 production in C3H/HeN mice. Besides, the production of IL-4 by splenic T cells derived from SKNS-treated C3H/HeN mice specific for OVA was significantly decreased compared with that in control mice. In contrast, there was no difference between production levels of OVA-specific serum Igs or splenic cytokines production in C3H/HeJ mice treated with SKNS and those not treated with SKNS. After nasal inhalation of OVA, counts of sneezing, eosinophilic infiltration and IL-5 expression in nasal mucosa were significantly decreased in SKNS-treated C3H/HeN mice. However, no significant differences were seen in C3H/HeJ mice treated with SKNS and those not treated with SKNS. These findings indicate that SKNS has an inhibitory effect in a murine allergic rhinitis model by enhancing IL-12 production from macrophages via TLR4.

Traditional medicines may influence TLR4 expression on macrophages by enhancing Th1 responses via up-regulation of IL-12 production. In the present study, we confirmed that SKNS up-regulates TLR4 gene expression on macrophages in vitro and in vivo. Furthermore, pretreatment with SKNS in vivo enhanced IL-12 production by macrophages following stimulation with LPS. Besides, SKNS inhibited Th2 responses and the allergic phenomenon in the murine allergic rhinitis model. In contrast, Th1 responses were up-regulated by SKNS treatment. Th2 response is inhibited by IFN-γ -producing Th1 cells. Macrophage/dendritic cell-derived cytokines such as IL-12, IL-15 and IL-18 are at least partly responsible for early IFN-γ production from NK cells and consequently Th1 cell differentiation. In our allergic rhinitis model, induction phase was achieved by intraperitoneal injection of OVA, not in nasal cavity, and this stimulation could be effective to macrophages or DCs because they are able to traffic to the nasal mucosa. 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 SKNS reduced Th2 responses in a murine allergic rhinitis model via up-regulation of TLR4 gene expression at least on macrophages. Our results thus offer a new approach using SKNS for the treatment of allergic disorders such as allergic rhinitis. Further studies are needed to elucidate the mechanisms of up-regulation of TLR4 gene expression by SKNS.

論文審査及び最終試験又は学力の確認の結果の要旨

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論文審査の結果の要旨

申請者は、アレルギー性鼻炎に対する漢方製剤千金内托散の効果を確認する目的で、マウスの培養細胞、および卵白アルブミン（Ovalbumin：OVA）を抗原としたマウスアレルギー性鼻炎モデルを用いて千金内托散投与の影響を検討した。マウスマクロファージRaw264.7細胞及び千金内托散を経口投与したマウスの腹腔マクロファージにおけるToll-like receptor 4（TLR4）とinterleukin（IL)-12 の発現を測定した。TLR4伝達シグナルの低下したマウスC3H/HeJ及びその野生型C3H/HeNを用いて、OVA誘発アレルギー性鼻炎モデルを作製し、千金内托散投与の影響を検討した。その結果、千金内托散投与により培養細胞のTLR4の発現増強とIL-12産生亢進作用が確認された。千金内托散投与により、C3H/HeNアレルギー性鼻炎モデルでは、血清中OVA特異的IgEとIgG1、脾細胞からのIL-4産生抑制とinterferon-γ産生亢進、鼻アレルギー症状の抑制、鼻粘膜組織のIL-5や好酸球の低下を認めたが、C3H/HeJアレルギー性鼻炎モデルでは見られなかった。以上から千金内托散はTLR4分子を介して鼻アレルギーを抑制することが示された。本研究は、漢方製剤による鼻アレルギー治療の可能性を示したものであり、臨床的意義も高く学位授与に値すると判断した。

最終試験又は学力の確認の結果の要旨

申請者は、漢方製剤千金内托散の効果を確認する目的で、マウス鼻アレルギーモデルを用いてその影響を解析し、TLR4を調節することでアレルギー性鼻炎を抑制することを示した。関連した知識も十分あり学位授与に値すると判断した。（主査：森田栄伸）

申請者は、マウスのアレルギー鼻炎モデルを用いて、漢方薬である千金内托散がTLR4シグナルを介してマクロファージのIL-12産生を亢進することによりTh1優位の免疫応答を誘導し、アレルギー反応の誘導期を抑制することを明らかにした。関連知識も豊富であり、学位授与に値すると判断した。（副査：原田　守）

申請者は、アレルギー反応における千金内托散の作用を検討し、マウスマクロファージのTLR4遺伝子発現を増強し、IL-12産生を亢進させること、アレルギー性鼻炎モデルでTLR4依存性にTh1応答を増強し、症状を緩和することを明らかにした。関連分野においても豊富な学識を有しており学位授与に値すると判断した。（副査：熊倉俊一）

（備考）要旨は、それぞれ４００字程度とする。