Suppressive effects of *Houttuynia cordata* Thunb (Saururaceae) extract on Th2 immune response

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Abstract

*Ethnopharmacological relevance:* *Houttuynia cordata* Thunb (Saururaceae), known as ‘E-Sung-Cho’ in Korea, has been traditionally used for the treatment of herpes simplex, chronic sinusitis, and allergy.

*Aim of the study:* To investigate the inhibitory activity of *Houttuynia cordata* Thunb fractions (HcFs) on the T helper 2 (Th2) immune response, we evaluated the alternation of the release of Th2-type cytokines and chemokines such as interleukin (IL)-4 and IL-5, and thymus and activation-regulated chemokine (TARC/CCL17).

*Materials and methods:* Ethanol fraction was obtained from dried and powdered whole plants of *Houttuynia cordata* Thunb using ethanol. The residue was diluted with water and was then successively partitioned with \(n\)-hexane, EtOAc, BuOH and water fractions. RT-PCR and ELISA were performed to measure mRNA and protein expression of cytokines.

*Results:* HcFs inhibited the expression of IL-4 and IL-5 in response to phorbol 12-myristate 13-acetate (PMA) and calcium ionophore (CaI) in Jurkat T cells and the human mast cell line, HMC-1. IL-4- and tumor necrosis factor-alpha (TNF-\(\alpha\))-induced TARC production was blocked by HcFs in skin fibroblast CCD-986sk cells, particularly by the ethanol extract of Hc. Stimulants included in PMA, phytohemagglutinin (PHA) and CaI, increased the mRNA level of CC chemokine receptor 4 (CCR4), a receptor of TARC, in Jurkat T cells, and the ethanol extract of HcF weakly blocked the increased mRNA level. However, the stimulants and ethanol extract had no effect on the CCR4 protein level. The ethanol extract inhibited TARC-induced migration, as well as basal migration of Jurkat T cells.

*Conclusions:* This study may show the usefulness of HcFs in the ethnopharmacological treatment of Th2-mediated or allergic inflammation, through the down-regulation of the production of Th2 cytokines and TARC, as well as cell migration.

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*Keywords:* *Houttuynia cordata* Thunb (Saururaceae); Th2-type cytokine; Allergic inflammation; Thymus and activation-regulated chemokine (TARC)

1. Introduction

Allergic inflammation is caused by the breaking of the Th1/Th2 balance, or by the induction of a Th2 lymphocyte-dominant immune response, which shows the pronounced accumulation of Th2 cells at the inflammatory sites of individuals with atopic dermatitis and asthma (Van Reijsen et al., 1992; Robinson et al., 1992). Th2 cells produce IL-4, IL-5, IL-10, and IL-13, known as Th2 cytokines, and the cytokines function as important factors in these disease processes (Heijink and Van Oosterhout, 2005; Homey et al., 2006). IL-4 plays a major role in B-cell activation and isotype switching, resulting in the generation of IgE antibodies (Paul, 1991). The control of IL-5 production is a key mechanism in the production of eosinophils in a wide range of diseases, especially helminth infections and allergies (Rothenberg and Hogan, 2006). Recent studies have
suggested that the migration of Th2 cells could be regulated by CC chemokines and their receptors (Bleul and Boehm, 2000). Thymus and activation-regulated chemokine (TARC) is known to be one of the CC chemokines, and its interaction with CC chemokine receptor 4 (CCR4) initiates the migration of Th2 cells in allergic inflammation because CCR4 is selectively expressed on Th2 cells (Imai et al., 1996, 1999). The serum TARC level in patients with AD was higher than that in patients with psoriasis, and it was found to be correlated with disease severity (Kakinuma et al., 2001). Human epidermal keratinocytes and skin fibroblast cells function as major sources of TARC production by inflammatory or Th2-type cytokines such as IL-1β, IFN-γ, TNF-α, and IL-4 (Vestergaard et al., 2001; Lee et al., 2006). These cytokines are the predominant cytokines that are secreted in chronic skin lesions in human atopic dermatitis, and induce regional production of TARC in the skin. This response contributes to the worsening of allergic inflammation (Vestergaard et al., 1999; Leung, 2000; Kakinuma et al., 2001; Vestergaard et al., 2001).

Houttuynia cordata Thunb (Houttuynia cordata) is known as ‘E-Sung-Cho’ in Korea, and is widely distributed throughout Southeast Asia. Many papers have reported the anti-viral activity, anti-cancer activity, and anti-allergic actions of Houttuynia cordata (Chang et al., 2001; Chiang et al., 2003; Li et al., 2005; Lu et al., 2006). It has recently been reported that Houttuynia cordata may be beneficial for the treatment of mast cell-mediated inflammation (Kim et al., 2007). However, the effects of Houttuynia cordata fractions (HcFs) on the release of Th2-type cytokines and TARC have not been studied completely, nor has the chemotactic activity observed in the associated cells. The aim of this study is to evaluate the anti-Th2-type allergic effects of HcFs by examining alternation of IL-4, IL-5, TARC, and CCR4.

2. Materials and methods

2.1. Preparation of fractionations from Houttuynia cordata

The whole plants of Houttuynia cordata (Saururaceae) were collected in Yeosu city, Jeonnam, Republic of Korea, in October 2004, and were identified by a botanist, Professor Joo-Hwan Kim. A voucher specimen (number 81) and the standard extract were deposited at the Herbarium of the Department of Life Science, Daejeon University (TUT), Republic of Korea. Thirteen g of whole plants of Houttuynia cordata (30 g) were extracted with EtOH (3 × 0.5 L) for 2 days at room temperature. The combined extracts (3.45 g) were concentrated under reduced pressure. The residue was diluted with water (0.25 L), and then successively partitioned with n-hexane (3 × 0.25 L), EtOAc (3 × 0.25 L), and BuOH (3 × 0.25 L) to yield n-hexane (1.25 g), EtOAc (0.96 g), BuOH (0.48 g), and water-soluble fraction (0.62 g), respectively. HcFs include EtOH, n-hexane, EtOAc, BuOH, and water-soluble fraction.

2.2. Reagents

Dulbecco’s modified Eagle’s medium (DMEM), RPMI-1640, antibiotics, and fetal bovine serum (FBS) were obtained from Invitrogen Life Technologies (Gaithersburg, MD). Human recombinant TNF-α, IL-4, and TARC/CCL17 were obtained from PeproTech (Rocky Hill, NJ). The ImProm-II reverse transcription system kit for isolation of total RNA and the PCR Master Mix were obtained from Promega (Madison, WI). PMA, PHA, and calcium ionophore (A23187; Cal) were obtained from Sigma (St. Louis, MO). Normal mouse IgG was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-CCR4 antibodies were obtained from R&D Systems (Minneapolis, MN).

2.3. MTT assay

Human Jurkat T cells and normal human skin fibroblast CCD-986sk cells were purchased from the American Type Culture Collection (Rockville, MD), and HMC-1 was supplied by Dr. Dong-Hee Kim (Daejeon University). Cell viability was determined using an MTT assay kit (Roche, Penzberg). In brief, 100 μL of Jurkat T cells (1 × 10⁶ cells/mL), HMC-1 cells (1 × 10⁶ cells/mL), and human skin fibroblast CCD-986sk cells (4 × 10⁵ cells/mL) were seeded in 96-well plates and incubated for 48 h after treatment with various concentrations (0, 1, 10, and 100 μg/mL) of HcFs, including EtOH, n-hexane, EtOAc, BuOH, and water-soluble fraction. Ten microlitres of MTT solution (0.5 mg/mL) was added, and the cells were incubated at 37°C for 4 h. One hundred microlitres of solubilization solution was then added to each well. After 24 h incubation, the optical densities of 96-well culture plates were measured using a spectrometer (Bio-Tek Instruments, VT) at 540 nm. The optical density of untreated control cells was taken as 100% viability.

2.4. Determination of IL-4, IL-5, IFN-γ, and TARC concentrations by ELISA

After treatment with PMA and Cal in Jurkat T cells and HMC-1 cells, and IL-4 and TNF-α in CCD-986sk cells, the concentrations of IL-4, IL-5, and IFN-γ in the supernatant were measured by a sandwich enzyme-linked immunosorbent assay (ELISA) using an OptEIA Set. IL-4, IL-5, and IFN-γ (BD Biosciences, San Diego, CA) and TARC concentrations were evaluated using an ELISA kit (R&D Systems) according to the instructions of the manufacturer. This ELISA method enabled the detection of TARC concentrations greater than 15.6 pg/mL, and data are presented as picograms of chemokine per 4 × 10⁵ cells.

2.5. RT-PCR

RT-PCR was performed for RNA detection of TARC and CCR4 mRNA in CCD-986sk cells and Jurkat T cells. After treatment with IL-4 and TNF-α in Jurkat T cells and PMA/PHA/Cal in CCD-986sk cells, total RNA was extracted from the cells, and was then subjected to reverse transcription using an ImProm-II reverse transcription system kit. The sequences of the PCR primers were as follows: TARC sense, 5′-
Fig. 1. The cytotoxic effects of HcFs on Jurkat T cells, HMC-1 cells, and human skin fibroblast CCD-986sk cells. Cell viability was evaluated by MTT assay 48 h after treatment with HcFs such as EtOH, n-hexane, EtOAc, BuOH, and water-soluble fractions at the indicated concentrations in Jurkat T cells (A), HMC-1 cells (B), and skin fibroblast CCD-986sk cells (C). Data represent the means ± S.D. of three independent experiments.

ACTGCTCCAGGGATGCCATCGTTTTT-3′; TARC antisense, 5′-ACA AGG GGA TGG GAT CTC CCT CAC TG-3′; CCR4 sense, 5′-CTT CCT GCC CCC ACT GTA TT-3′; CCR4 antisense, 5′-TCT TCA CCG CCT TGT TCT TC-3′; and GAPDH sense, 5′-ACC ACA GTC CAT GCC ATC AC-3′; GAPDH antisense, 5′-TCC ACC ACC CTG TTG CTG TA-3′. These primers yielded PCR products of the expected sizes of 270 bp for TARC, 608 bp for CCR4, and 452 bp for GAPDH. The PCR protocol was comprised of an initial denaturation step at 95 °C for 30 s, followed by 35 cycles of amplification. For the amplification of TARC and CCR4 cDNA, the cycles consisted of denaturation at 95 °C for 1 min, annealing at 58 °C for 1 min, and elongation at 72 °C for 1 min. For the amplification of GAPDH cDNA, the cycles included denaturation at 95 °C for 1 min, annealing at 56 °C for 1 min, and elongation at 72 °C for 1 min. To verify the specificity of the amplification, we also subjected PCR products to electrophoresis on a 1% agarose gel, which was then stained with ethidium bromide and examined with a Nighthawk system (Huntington Station, NY). GAPDH was used as an internal control in each sample.

2.6. Chemotaxis assay

Cell migration assay was performed using a 48-well microchamber (Neuroprobe, Gaithersburg, MD). The lower wells were filled with 28 μL buffer alone or with buffer that contained TARC and a polyvinylpyrrolidone-free filter (Neuroprobe) with a 5-μm pore-size was placed over the lower well. The membranes were pre-coated with RPMI 1640 that contained fibronectin (100 μg/mL) for overnight at 4 °C. The upper wells were filled with 50 μL of Jurkat T cells at 5 × 10⁶ cells/mL in the medium containing 1% BSA and 30 mM HEPES. The chamber was incubated for 3 h at 37 °C. After the polycarbonate filter was removed, the cells adhering to its upper surface were wiped off with a filter wiper. The filter was dried, fixed, and stained with Diff-Quick (Baxter, McGaw Park, IL). The cells of at least two randomly selected fields per well were counted by using an Axiovert 25 (Carl Zeiss, Jena, Germany) and the Visus Image Analysis System (Foresthill Products, Foresthill, CA). The chemotactic index (CI) was calculated from the number of cells that had migrated as compared to the control.

3. Results

3.1. The cytotoxic effects of HcFs on Jurkat T cells, HMC-1 cells, and human skin fibroblast cells

We performed an MTT assay to test the cytotoxicity of HcFs in Jurkat T cells, HMC-1 cells, and human skin fibroblasts. Fig. 1

Fig. 2. Inhibition of IL-4 and IL-5 production by HcFs in Jurkat T cells. The cells were pre-treated in the absence or presence of 10 μg/mL HcFs such as EtOH, n-hexane, EtOAc, BuOH, or water-soluble fractions for 1 h, and were then challenged with 50 ng/mL PMA and 1 μM Cal for 24 h. The supernatant was analyzed by ELISA. Data are expressed as the means ± S.D. of three independent experiments, and are presented as the percentage of cytokine concentration (the cytokine level in the PMA/Cal-treated cells was set at 100%). *p < 0.05 and **p < 0.01 are assessed as significant differences between the untreated group and the PMA/Cal-treated group or between the PMA/Cal-treated group and each of the HcFs-treated groups.
3.2. Inhibition of IL-4 and IL-5 production by HcFs in Jurkat T cells and HMC-1 cells

Since T cells and mast cells function as essential cells in IL-4 and IL-5 production, we examined the alternation of Th2 cytokines in response to pre-treatment of Jurkat T cells and HMC-1 cells with HcFs. It has been reported that activation of T cells and mast cells is achieved by treatment with PMA and Cal (Mordvinov et al., 1999; Zhao et al., 2004). As shown in Figs. 2 and 3, treatment with PMA and Cal augmented the productions of IL-4 and IL-5 in Jurkat T cells and HMC-1 cells. This increase was suppressed by HcFs, and IL-5 expression was strongly inhibited by HcFs such as EtOH, n-hexane, EtOAc, BuOH, and water-soluble fraction, despite the differences in inhibition by respective fractions and cells. However, the Th1-type cytokine, IFN-γ, was not affected by the HcFs.

3.3. Inhibition of TARC secretion by HcFs in skin fibroblast cells

In a previous paper, our group reported that skin fibroblast cells increase the expressions of chemokines and cytokines as a result of treatment with IL-4 and TNF-α (Lee et al., 2006). In this experiment, we confirmed the increase of TARC production due to TNF-α and IL-4 in normal human skin fibroblast CCD-986sk cells using ELISA (Fig. 4A). HcFs suppressed the up-regulation of TARC, while the EtOH fraction of the HcFs strongly inhibited the mRNA and protein expression of TARC in a dose-dependent manner (Fig. 4B and C).
Fig. 5. Effect of EtOH fraction of HcFs on CCR4 expression in Jurkat T cells. (A) The cells were stimulated in the absence or presence of 10 µg/mL PHA, 10 ng/mL PMA, or 100 nM CaI, and were incubated at 37 °C for 24 h. The mRNA expression of CCR4 was measured by semi-quantitative RT-PCR. (B) The cells were pre-treated at the indicated concentration of the respective EtOH fraction of HcFs for 1 h, and were then stimulated with 10 µg/mL PHA, 10 ng/mL PMA, and 100 nM CaI. The cells were incubated at 37 °C for 24 h, and the mRNA expression of CCR4 was then measured by semi-quantitative RT-PCR. The amount of CCR4 mRNA was normalized using GAPDH mRNA. (C) The cells were pre-treated in the absence or presence of 100 µg/mL of the EtOH fraction of HcFs for 1 h, and were then stimulated with 10 µg/mL PHA, 10 ng/mL PMA, and 100 nM CaI. The cells were incubated at 37 °C for 48 h and analyzed by a fluorescence-activated cells sorter using monoclonal anti-CCR4 antibodies. A baseline level was obtained by incubating the cells with normal mouse IgG. Data are expressed as the representative results of three individual experiments.

3.4. Effect of the EtOH fraction of HcFs on the chemotactic activity of Jurkat T cells

Since the EtOH fraction inhibited TARC expression, we then tested the effect of the extract on the expression of CCR4, a TARC receptor. As shown in Fig. 5A and B, the mRNA expression of CCR4 was induced by PMA/PHA/CaI treatment, and was inhibited by the EtOH fraction extract. Although the CCR4 protein was weakly expressed on the surface, it was not altered by treatment with PMA/PHA/CaI or the EtOH fraction (Fig. 5C). Before testing the effect of the EtOH fraction on the migration of Jurkat T cells, we performed a chemotaxis assay in response to TARC. Cell migration peaked at a TARC concentration of 100 ng/mL (Fig. 6A). PMA/PHA/CaI treatment blocked the cell movement, and pre-treatment with the EtOH fraction more strongly inhibited cell movement in a dose-dependent manner (Fig. 6B). To investigate whether the inhibitory effect of the EtOH fraction is associated with PMA/PHA/CaI treatment, we examined both basal and TARC-responding chemotaxis after treatment with only the EtOH fraction. Surprisingly, the EtOH fraction completely blocked basal cell migration, as well as TARC-induced cell movement (Fig. 6C).

Fig. 6. Effect of EtOH fraction of HcFs on chemotactic activity of Jurkat T cells. (A) Jurkat T cell migration in response to the indicated concentration of TARC was measured by chemotaxis assay. Data are expressed as the mean ± S.D. (B) The cells were pre-treated in the absence or presence of the indicated concentration of EtOH fraction of HcFs for 1 h, and were then stimulated with 10 µg/mL PHA, 10 ng/mL PMA, and 100 nM CaI. The cell migration in response to 100 ng/mL TARC was measured by chemotaxis assay. The data are expressed as means ± S.D. in relation to the negative control, which was set at 100%. **p < 0.01 is assessed as a significant difference between the untreated group and the PMA/PHA/CaI-treated group or between the PMA/PHA/CaI-treated group and the EtOH fraction-treated group. (C) The cells were pre-treated in the absence or presence of 100 µg/mL of the EtOH fraction of HcFs for 1 h, and were then stimulated with 10 µg/mL PHA, 10 ng/mL PMA, and 100 nM CaI. The cell migration in response to PBS or 100 ng/mL TARC was measured by chemotaxis assay. The data are expressed as the means ± S.D. in relation to the negative control, which was set at 100%. **p < 0.01 is assessed as a significant difference between the untreated group and the EtOH-treated group or between the TARC-treated group and the TARC/EtOH fraction-treated group.
4. Discussion and conclusion

Allergic diseases are caused by an aberrant immune response that is mediated by effector cells, Th2 cells, and the associated cytokine pattern, which includes IL-4, IL-5, and IL-13 (Heijink and Van Oosterhout, 2005; Homey et al., 2006). Therefore, the most pronounced finding on allergy therapy is directly related to the control of these Th2 immune effectors. In the present study, we investigated the inhibitory effect of an herb, Houttuynia cordata, on both the Th2-mediated and the TARC-related response for the first time.

Houttuynia cordata, referred to as ‘E-Sung-Cho’ in Korea, has been well known to have therapeutic activity on herpes simplex, chronic sinusitis, allergies, and cancer (Rho, 1998; Bae et al., 2001; Chiang et al., 2003; Lu et al., 2006). We first examined the cytotoxicity of HcFs on Jurkat T cells, HMC-1 cells, and human fibroblast CCD-986sk cells at concentration range from 1 μg/mL to 100 μg/mL. HcFs had no cytotoxic effect at a concentration of 10 μg/mL, but did have a partial effect at a concentration of 100 μg/mL (Fig. 1). Although Th2 cytokines are produced by a variety of cell types, the production by activated T cells is key to the pathogenesis of allergic diseases. Mast cells also release the cytokines implicated in the immediate-type hypersensitivity reaction, which include IL-3, IL-4, IL-6, and IL-13, and TNF-α (Bradling et al., 1994). This finding is compatible with the traditional view that mast cells are implicated in the immediate reaction. IL-4 promotes the production of IgE, and IL-5 accelerates the production of eosinophils in the bone marrow (Paul, 1991; Rothenberg and Hogan, 2006). By contrast, Th2 cells do not produce Th1 cytokines, IL-2, or IFN-γ. These cytokines are characteristic of Th1 cells, which, reciprocally, do not express Th2-type cytokines (Chatila, 2004). As shown in Figs. 2 and 3, HcFs inhibited IL-4 and IL-5 secretion induced by PMA and Cal in Jurkat T cells and HMC-1 cells. However, IFN-γ was not suppressed by HcFs. These results indicate that HcFs show potential as therapeutic materials for the treatment of allergic inflammation.

Both TARC and CCR4 expression essentially induces a dominant Th2 response during allergic inflammation (Bisset and Schmid-Grendelmeier, 2005). Because TARC is mainly produced in fibroblast cells and CCR4 expression plays an important role in T cells, we examined whether HcFs affect the expression of TARC in human fibroblast CCD-986sk cells and the expression of CCR4 in Jurkat T cells. We previously showed that the combination of TNF-α and IL-4 induces a synergistic increase in the expression of TARC at both the protein and mRNA levels in human skin fibroblast cells (Lee et al., 2006). Data shown in Figs. 4 and 5 demonstrate that HcFs inhibited the increase of the mRNA and protein of TARC. Although HcFs decreased the elevated mRNA expression of CCR4 induced by PMA/PHA/Cal, they did not affect the expression of the CCR4 protein. These results may be caused by a slight amount of basal CCR4 expression or weak translation of CCR4 mRNA. We then investigated the chemotactic activity resulting from the interaction of TARC and CCR4. The peak chemotactic index in Jurkat T cells appears upon treatment with a 100 ng/mL concentration of TARC (Fig. 6A). PMA/PHA/Cal treatment unexpectedly inhibited the migration of cells, and the EtOH fraction was also decreased in a dose-dependent manner (Fig. 6B). Because the PMA/PHA/Cal stimulation blocked proliferation and increased cell adhesion, we considered that these results have no association with the alternation of CCR4 expression and the effect of the EtOH fraction in PMA/PHA/Cal. We then examined the direct effect of the EtOH fraction. The EtOH fraction lowered basal cell movement, reflecting TARC-induced migration. We supposed that the EtOH fraction down-regulates the chemotaxis induced by a variety of chemokines rather than TARC-specific migration.

In regard to the mechanism of HcFs for the treatment of atopic dermatitis, asthma, and nasal allergies, this study demonstrated that they have potent inhibitory effects on important allergy-related cytokines and chemokines released from the human lymphocytes, fibroblasts, or mast cells, and blocked basal chemotactic activity, as well as TARC-induced cell migration. In further study, we will examine the functions of respective HcFs and compare the inhibitory effects among the fractions, as well as the suppressive effects of HcFs on chemotaxis in response to other chemokines.

In summary, HcFs down-regulate the expressions of IL-4, IL-5, TARC, and CCR4 receptor, but do not have the same effect on IFN-γ. These results indicate the beneficial therapeutic effects of HcFs on Th2-mediated or allergic skin disorders, and support a further understanding of the mechanisms of natural compounds in the treatment of allergies.

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