The inhibitory effect of *Houttuynia cordata* extract on stem cell factor-induced HMC-1 cell migration

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Abstract

Hottuynia cordata Thunb (Saururaceae; HC) is known as a therapeutic drug that has been used in traditional oriental medicine for the treatment of allergy. Mast cells play an important role in a variety of inflammatory diseases, and specifically asthma and atopy. In the present study, we investigated the effect of HC extracts on the migration of the human mast cell line, HMC-1, in response to stem cell factor (SCF). Treatment with HC extracts at a concentration of 10 μg/ml for 24 h showed no significant decrease in the survival rate of the HMC-1 cells. SCF showed the typical bell-shape curve for the HMC-1 cell chemoattraction with the peak of the curve at the SCF concentration of 100 ng/ml. HC-1, which was the whole plant (*Houttuynia cordata*) extracted with 80% EtOH, and HC-3, which was the residue successively partitioned with EtOAc, both had inhibitory effects on HMC-1 cell movement. After the treatment with 10 μg/ml HC-1 extract for 6 and 24 h, the chemotactic index (CI) of HMC-1 cells decreased up to 74 and 63%, respectively. HC-3 extract treatment for 6 and 24 h lowered the CI to 72 and 44%, respectively. The HC-1 and HC-3 extracts had no inhibitory effect on the mRNA and surface protein expressions of c-kit, SCF receptor. SCF mediated the chemotaxis signaling via NF-κB activation, and both extracts inhibited the activation. Therefore, our results indicate that HC-1 and HC-3 extracts decrease the chemotactic ability of HMC-1 cells in response to SCF by inhibiting the NF-κB activation, and these substances may be useful for treating mast cell-induced inflammatory diseases.
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Keywords: *Hottuynia cordata*; Mast cells; Cell migration; NF-κB

1. Introduction

Hottuynia cordata Thunb (Saururaceae; HC) is a perennial herb native to Southeast Asia, and it has a thin stalk and heart-like leaf. It is called E-Sung-Cho in Korea, and it is known to be an effective drug for treating allergic inflammation. Recent reports have demonstrated that HC is effective for treating anaphylaxis,

cancer and viral infection (Chiang et al., 2003; Kwon et al., 2003; Li et al., 2005; Lu et al., 2006).

Mast cells (MCs) play an essential role in allergic inflammation, including asthma, atopic dermatitis and allergic rhinitis (Williams and Galli, 2000; Theoharides and Cochrane, 2004). MCs originate from hemopoietic stem cells and their maturation is completed in the connective tissue. MCs migrate toward the infected tissues after activation by an antigen. Cell movement of leukocytes as well as that of the MCs is a very critical step in the inflammatory response. Infiltrated MCs secrete a variety of pro-inflammatory mediators such as histamine, cytokines and prostaglandin, and this results in the genesis of the disease process (Bisset and Schmid-Grendelmeier, 2005).

Stem cell factor (SCF) is a ligand for c-kit, which is the receptor tyrosine kinase. SCF is a major effector that promotes cell

proliferation, survival, differentiation, adhesion and functional activation, and particularly for the mast cell lineage (reviewed in Ref. Ashman, 1999). SCF is a chemotactic factor for MCs and hemopoietic stem cells (Nilsson et al., 1994; Okumura et al., 1996). It has been reported that the SCF expression is associated with mast cell-associated diseases such as allergic rhinitis and nasal polyposis (Kim et al., 1997; Otsuka et al., 1998). A recent study has been demonstrated that both SCF and c-kit expressions are involved in asthma (Al-Muhsen et al., 2004). After SCF binding, the c-kit dimerizes, autophosphorylates and then transduces multiple signaling pathways, depending on the different cell types (Linnekin, 1999). These mechanisms include the JAK/STAT pathway, the RAS-RAF-MAP kinase cascade and the phosphatidylinositol 3-kinase (PI3K)/Akt pathway.

Although it has been shown that Lyn is required for SCF-induced chemotaxis of primary hematopoietic cells, a precise mechanism of SCF-induced migration in MCs is not well understood (O'Laughlin-Bunner et al., 2001). Therefore, in the present study, we investigated whether HC has anti-inflammatory effect on MCs via the inhibition of cell migration. Furthermore, we studied the SCF-induced signal for chemotaxis and we then determined the contribution of HC to this mechanism.

2. Materials and methods

2.1. Materials and cell culture

Fetal bovine serum (FBS), and Trizol were purchased from Life Technologies Inc. (Gaithersburg, MD). Fibronectin was obtained from Sigma (St. Louis, MO). PP2, AG490, PD98059, SB202190, Ly294002 and SN50 were products of Calbiochem (San Diego, CA). Recombinant human stem cell factor was obtained from Peprotech (Rocky Hills, NJ). Antibodies against NF-κB, and ERK2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The human mast cell line HMC-1 was the kind gift of Dr. Butterfield (Rochester, MN), and they were grown in Iscove's medium supplemented with 10% heat-inactivated FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml).

2.2. Preparation of the HC extracts

The whole plants of *Houttuynia cordata* Thunb (Saururaceae) were collected in Yeosu city, Jeonnam, Korea, in October 2004. A voucher specimen (number 81) and the standard extract have been deposited at the Herbarium of the Department of Herbal Pharmaceutical Development, the Korean Institute of Oriental Medicine, Daejeon, Korea and the Division of Life Science, Daejeon University (TUT), Korea. The dried and powdered whole plants of *Houttuynia cordata* (30 g) were extracted with 80% EtOH (3×0.5 l) for 2 days at room temperature. The combined extracts (3.45 g; HC-1) 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 produce the *n*-hexane (1.25 g; HC-2), EtOAc (0.96 g; HC-3), BuOH (0.48 g;

HC-4), and the water-soluble fractions (0.62 g; HC-5), respectively.

2.3. MTT assay

We performed MTT assay to determine the cell viability with using the MTT assay kit (Roche, Penzberg, Germany). The 5×10^4 HMC-1 cells in 100 μl of the culture medium were dispensed onto a 96-well culture plate. Each HC extract was added to the individual wells to achieve a final concentration of 10 μg/ml. The plate was then incubated for 24 h at 37 °C in a CO_2 incubator. After the addition of 10 μl of MTT solution in each well, the plate was incubated at 37 °C for 4 h in a CO_2 incubator. The 100 μl of solubilization solution was then added to each well. After 24 h incubation, the absorbance was measured by using an ELISA reader (Bio-Tek Instruments, VT) at 550 nm.

2.4. Chemotaxis assay

Cell migration 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 SCF and a polyvinylpyrrolidone-free filter (Neuroprobe) with an 8 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 HMC-1 cells at 5×10^6 cells/ml in Iscove's medium that contained 1% BSA and 30 mM HEPES. The chamber was incubated for 5 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.

2.5. Semi-quantitative RT-PCR

RT-PCR was performed to determine the relative quantity of c-kit mRNA in the HC extract-stimulated HMC-1 cells. The total RNA was extracted from the cells using Trizol reagent as described by the manufacturer's instructions. For preparation of the cDNA, the total RNA (2 μg) was incubated at 37 °C for 90 min with using a first-strand cDNA synthesis kit (Promega, Madison, WI). The cDNAs were denatured at 94 °C for 5 min and then they were amplified by 30 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min and extension at 75 °C for 1 min, and a final extension step was done at 75 °C for 5 min. The primers used in this study are as follows: c-kit; AAA GGA GAT CTG TGA GAA TAG GCT C and AGC TCC CAA AGA AAA ATC CCA TAG G, GAPDH; ACC ACA GTC CAT GCC ATC AC and TCC ACC ACC CTG TTG CTG TA. GAPDH was used as an internal control for each PCR reaction. The final PCR products were separated on 1% agarose gel, and then they were visualized with ethidium bromide staining.

2.6. Western blot analysis

The HMC-1 cells seeded into a 6-well plate at 5×10^5 cells/ml. After treating them with SCF or HC extract, the cells were harvested and then lysed in cytosolic extraction buffer (10 mM HEPES, 10 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 1% NP-40, 0.1 mM dithiothreitol (DTT), 0.1 mM Na_3VO_4 and protease inhibitors). The homogenate was centrifuged at $10,000 \times g$ for 1 min at $4\,^{\circ}C$. The supernatant was collected as a cytosolic fraction. The pellet was resuspended in 50 μl of nuclear extraction buffer (20 mM HEPES, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 25% glycerol, 1 mM DTT, 0.1 mM Na_3VO_4 and protease inhibitors) and it was centrifuged at $12,000 \times g$ for 15 min at $4\,^{\circ}C$. The supernatant was collected as a nuclear fraction. The protein samples (50 μg/lane) were separated by SDS-polyacrylamide gel electrophoresis (10%), and the proteins were then transferred onto nitrocellulose filters. The blots were incubated with anti-NF-κB antibodies and they were developed with the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech.). The same blot was stripped and reprobed with anti-ERK2 antibodies for use as an internal control.

2.7. Statistical analysis

The data are expressed as means ± S.D. statistical differences were analyzed by using a paired t-test for a two-group comparison. The SPSS statistical software package (Version 10.0, Chicago, IL) was used for statistical analysis. p values < 0.01 were deemed statistically significant.

3. Results

3.1. The HC extracts have no cytotoxic effect on HMC-1 cells

Prior to examining the effect of HC extracts (HC-1-HC-5) on HMC-1 cells, we first examined whether the HC extracts affected the cell viability. As shown in Fig. 1, the survival rate of HMC-1 cells was not altered by treatments with the HC extracts at a concentration of 10 μg/ml for 24 h. In addition, the HC extracts at high concentration (100 μg/ml) for 24 h induced no decrease in the cell survival rate (data not shown).

3.2. The HC-1 and HC-3 extracts inhibit the HMC-1 cell migration in response to SCF

Because cell migration is a pivotal step in the inflammatory response, we investigated the alteration of HMC-1 cell migration after stimulation with the HC extracts. A chemotaxis assay was performed for measuring the HMC-1 cell movement. SCF induced the typical bell-shape curve for the HMC-1 chemoattraction and it showed the maximum activity at a concentration of 100 ng/ml (Fig. 2A). After pre-treatment with HC-1 extract for 6 and 24 h, cell migration in response to SCF showed a significant decrease of up to $73 \pm 5\%$ and $63 \pm 5\%$, respectively ($p < 0.01$) (Fig. 2B). Also, the HC-3 extract significantly inhibited the cell movement ($72 \pm 6\%$ and $44 \pm 2\%$) ($p < 0.01$). However, the
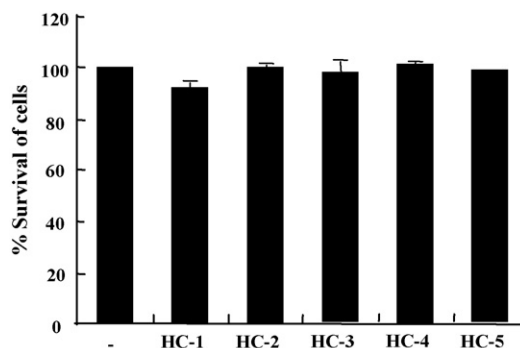

Fig. 1. HC extracts have no cytotoxic effect on HMC-1 cells. HMC-1 cells were incubated with the indicated HC extract at a concentration of 10 μg/ml for 24 h. The survival rate was measured by performing MTT assay as described in Section 2. The data are expressed as the relative ratio to the absorbance of the untreated HMC-1 cells, which was set at 100%. Three replicate measurements are included in a single experiment. The data are expressed as means ± S.D. of two independent experiments.
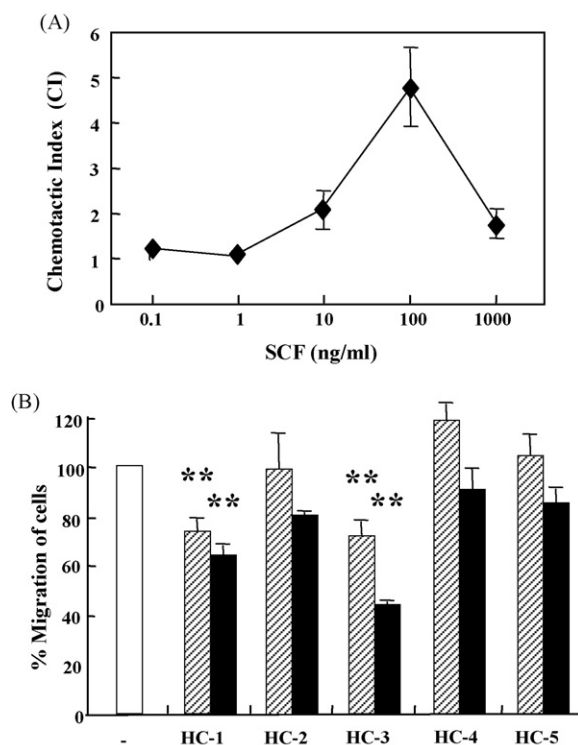

Fig. 2. HC-1 and HC-3 extracts inhibit the SCF-induced cell migration. (A) HMC-1 cells were placed in the indicated concentration of SCF in a 48-well microchamber, and they were allowed to migrate for 5 h. The number of cells that migrated was counted microscopically in three individual wells. Data are expressed as mean CIs ± S.D. (B) HMC-1 cells were pre-incubated in the absence (white bar) or presence of the indicated HC extract at a concentration of 10 μg/ml for 6 h (hatched bar) or 24 h (black bar). The cells were applied to 100 ng/ml SCF in a 48-well microchamber and they were allowed to migrate for 5 h. The number of cells that migrated was counted microscopically in six individual wells. The data are expressed as means ± S.D. in relation to the negative control, which was set at 100%. **$p < 0.01$ was accepted as a significant difference.

(A)

Stimulator	−	HC-1	HC-3
c-kit			
GAPDH			

(B)

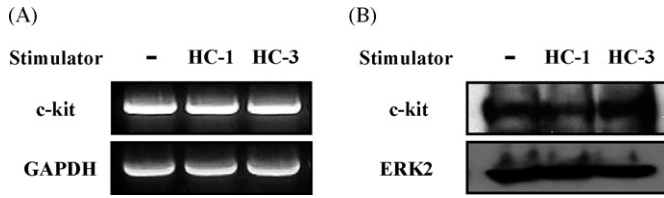| Stimulator	−	HC-1	HC-3
c-kit			
ERK2			

Fig. 3. The c-kit expression in HMC-1 cells is not altered by the HC-1 and HC-3 extracts. HMC-1 cells were serum starved with 0.5% serum for 24 h, and then they were incubated in the absence or presence of HC-1 or HC-3 extract at a concentration of 10 µg/ml for 24 h. The total RNA and protein was extracted and analyzed by semi-quantitative RT-PCR (A) or Western blotting (B) as described in Section 2. The bands were normalized with GAPDH or ERK2. The data are expressed as being representative of three individual experiments.

other extracts, HC-2, HC-4 and HC-5, had no inhibitory effect on cell migration.

3.3. The c-kit expression in HMC-1 cells is not altered by the HC-1 and HC-3 extracts

Because the HC-1 and HC-3 extracts inhibited HMC-1 migration in response to SCF, we investigated whether or not both extracts affect the mRNA and protein expressions of c-kit. Fig. 3 shows that the HC-1 and HC-3 extracts had no effect on the mRNA and protein expressions of c-kit. These results indicate that the HC-1 and HC-3 extracts inhibited cell migration by blocking the signaling molecule, which is involved in SCF-induced chemotaxis signaling, instead of inducing c-kit down-regulation.

3.4. NF-κB is involved in SCF-induced chemotaxis signaling in HMC-1 cells

To clarify the chemotaxis signaling induced by SCF, we examined the signaling molecules associated with this mech-
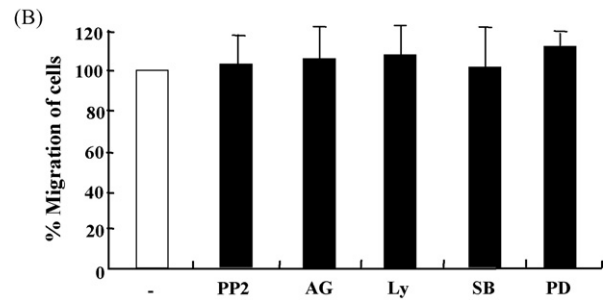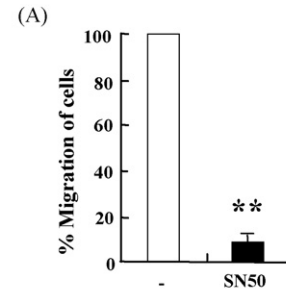
(A)

(B)

Fig. 4. NF-κB is involved in the SCF-induced chemotaxis signaling. The HMC-1 cells were pre-treated in the absence (white bar) or presence (black bar) of 18 µM SN50 (A), 10 µM PP2 (B, PP2), 20 µM AG490 (B, AG), 10 µM Ly294002 (B, Ly), 20 µM SB202190 (B, SB) or 20 µM PD98059 (B, PD) for 30 min. The cells were allowed to migrate for 5 h. The data are expressed as means ± S.D. of two independent experiments, and the data are presented in relation to the negative control, which was set at 100%. **$p < 0.01$ were accepted as significant differences.

anism by using pharmacological inhibitors. Pre-treatment with SN50, an inhibitor of NF-κB decreased the SCF-induced HMC-1 cell migration up to approximately 10% (Fig. 4A). This result means that NF-κB functions as an important signaling protein in HMC-1 cell migration. To determine the other signaling molecules that contribute to SCF-induced chemotaxis, we tested

(A)

	Cytosol						Nucleus					
Time	0	15m	30m	1h	2h	4h	0	15m	30m	1h	2h	4h
NF-κB												
ERK2												

(B)

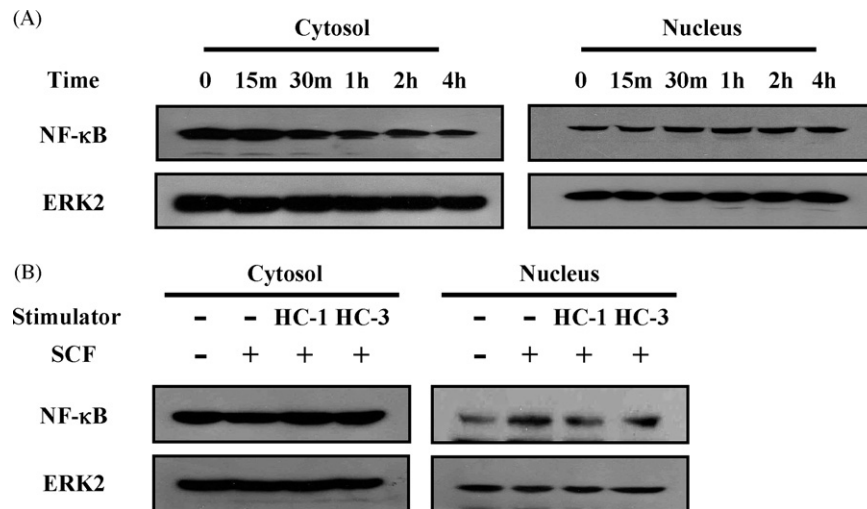|	Cytosol				Nucleus			
Stimulator	−	−	HC-1	HC-3	−	−	HC-1	HC-3
SCF	−	+	+	+	−	+	+	+
NF-κB								
ERK2								

Fig. 5. The HC-1 and HC3 extracts inhibit NF-κB activation. (A) Serum starved HMC-1 cells were stimulated with 100 ng/ml SCF for the indicated time. The harvested cells were lysed as described in Section 2, and the proteins were analyzed by Western blotting. The membrane was stripped and reprobed with anti-ERK2 antibodies as an internal control. (B) Serum-starved HMC-1 cells were pre-incubated in the absence or presence of 10 µg/ml of the HC-1 or HC-3 extract for 1 h, and then they were incubated with 100 ng/ml SCF for 2 h. The harvested cells were lysed as described in the methods section, and the proteins were analyzed by Western blotting. The membrane was stripped and reprobed with anti-ERK2 antibodies as an internal control.

the respective effects of the inhibitors of JAK (AG490), Src (PP2), PI3K (Ly294002), MEK (PD98059) and p38 MAPK (SB202190) on the chemotactic activity of SCF. However, the other signaling inhibitors had no significant effect on the migration of HMC-1 cells (Fig. 4B).

3.5. The HC-1 and HC3 extracts inhibit NF-κB activation

Since NF-κB is involved in SCF-induced chemotaxis signaling, we examined effects of the HC-1 and HC-3 extracts on NF-κB activation. As shown in Fig. 5A, SCF induced NF-κB translocation from a cytosol to a nucleus in a time-dependent manner. Both of the extracts blocked the NF-κB translocation from the cytosol (Fig. 5B). These results indicate that the HC-1 and HC-3 extracts inhibited cell migration in response to SCF by blocking the NF-κB activation.

4. Discussion and conclusion

HC has been widely used as an anti-allergic drug in traditional oriental medicine, and MCs function as key cells in the process of allergic inflammation. However, the exact effect of HC in MCs has not been well characterized. In this study, we investigated the effects of HC extracts on the SCF-induced cell migration of the human mast cell line, HMC-1. We demonstrated that (1) the HC-1 and HC-3 extracts decreased the HMC-1 cell movement in response to SCF, (2) the HC-1 and HC-3 extracts had no effect on the c-kit mRNA and protein expressions, (3) NF-κB was involved in the SCF-induced chemotaxis signaling and (4) the HC-1 and HC-3 extracts inhibited NF-κB translocation after SCF treatment.

Although the herb *Hottuynia cordata* Thunb has been used in traditional oriental medicine for the treatment of allergy and it is known to be a therapeutic drug, its exact mechanism remains to be determined. Recent studies have reported on the anti-allergic mechanism induced by this herb in both in vitro and in vivo models (Shin et al., 2003; Kim et al., 2004; Lee et al., 2005; Li et al., 2005). It has been reported that HC has inhibitory effects on anaphylactic reaction and mast cell activation (Li et al., 2005). SCF is a growth factor in both hematopoietic cells and germ cells, and it is produced in two forms, the soluble form, and the membrane-bound form by a variety of cells such as fibroblasts and epithelial cells (Wen et al., 1996; Zhang et al., 1996; Linnekin, 1999). It is also essential for the activation and chemotaxis of MCs, which is a critical step in an inflammatory response such as allergic inflammation (Nilsson et al., 1994; Schwartz, 1994).

We have investigated for the first time the inhibitory effect of HC extract on MC chemotaxis. Our results showed that the HC-1 and HC-3 extracts inhibited HMC-1 cell migration in response to SCF, and this effect was without any cellular toxicity (Figs. 1 and 2). To explain how the HC extracts blocked MC migration, we considered two possible mechanisms: (1) the HC extracts down-regulated the expression of c-kit, a receptor of SCF and (2) the HC extracts inhibited the activation or expression of the signaling proteins associated with HMC-1 chemotaxis. We first examined for any possible alteration of

the c-kit expression. The mRNA and protein expressions of c-kit were not regulated by the extracts (Fig. 3) and the result led us to determine the chemotaxis signaling induced by SCF. It has been reported that depending on cell type, SCF activates JAK, Src, PI3K, p38 MAPK, ERK and NF-κB (Linnekin, 1999; Coward et al., 2002). In chemotaxis experiments with using specific signaling inhibitor, SN50 uniquely inhibited the HMC-1 chemoattraction in response to SCF (Fig. 4). To confirm NF-κB's involvement in the SCF-induced chemotaxis signaling, we tested for activation of NF-κB by performing Western blotting. NF-κB translocates from a cytosol to a nucleus in a time-dependent manner after SCF treatment (Fig. 5A). Although we have not found the precise NF-κB-dependent signaling pathway, our results suggest that SCF mediated chemotaxis signaling via NF-κB activation. The HC-1 and HC-2 extracts inhibited the NF-κB translocation that was induced by SCF (Fig. 5B). The inhibition of HMC-1 cell migration that was induced by the extracts was weaker than that induced by SN50. This difference can be explained by the result that the extracts partially blocked NF-κB activation. The important result is that the HC-3 extract plays a dominant role in the inhibition of cell migration in response to SCF, as compared with the HC-1 extract (Fig. 2B). The HC-3 extract was fractionated from the HC-1 extract. Although we have not demonstrated the precise materials/proteins that were responsible for inhibiting chemotaxis signaling, and the HC extract included these materials/proteins, the fractionation process of the HC extract demonstrated the possibility of precisely finding more effective materials/proteins. Further investigations are now required, and our lab is now investigating this.

Until now, many researchers have presented a variety of targeted therapies for allergic diseases by using the mechanisms associated with MCs (Peachell, 2005). β_2 adrenoreceptor agonists are being used for reversing the bronchoconstriction of asthma by inhibition of the involved mediators. Antibodies against IgE, IL-4 and IL-5, which function as inhibitors of mast cell activation, are being developed as therapeutic drugs. A therapeutic treatment based on the mechanism of cell migration is one of the prospective therapies. Recent papers have shown the possibility of using the anti-allergic therapies of traditional oriental medicine (Li et al., 2005; Lu et al., 2006). This study on the effect of HC extracts and the chemotaxis signaling of MCs may be of significant benefit for treating allergic diseases.

In conclusion, we demonstrated that the HC-1 and HC-3 extracts decrease the nuclear translocation of NF-κB that is induced by SCF, and it results in decreased MC migration in response to SCF. HC extract may inhibit the inflammatory effect of MCs by regulating the chemotaxis signaling mediated by SCF. This study indicates the significance of HC extract as a possible drug for treating allergy.

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