

Short Communication

Shishihakuhito, a traditional Chinese medicine for atopic dermatitis, inhibits IgE-mediated histamine release from rat RBL-2H3 basophilic leukocyte cells

Mie WAKABAYASHI,^{a)} Eiko SAKURAI,^{b)} Kazuhiko YANAI,^{b)} Kazuhiro UMEMURA,^{a)} Masaaki YOSHIDA,^{c)} Yasushi OHIZUMI^{a,d,e,f)} and Tohru YAMAKUNI^{*a)#}

^{a)}Department of Pharmaceutical Molecular Biology, Graduate School of Pharmaceutical Sciences, Tohoku University, 6-3 Aoba, Aramaki, Aoba-ku, Sendai 980-8578, Japan. ^{b)}Department of Pharmacology, Tohoku University, Graduate School of Medicine, 2-1 Seiryomachi, Aoba-ku, Sendai 980-8575, Japan. ^{c)}Research Laboratory, Kotaro Pharmaceutical Co., Ltd, Ro 96-1 Kashima-machi, Hakusan, Ishikawa 920-0201, Japan. ^{d)}Yokohama College of Pharmacy, 601 Matano-cho, Tozuka-ku, Yokohama 245-0066, Japan. ^{e)}Research Center of Supercritical Fluid Technology, Graduate School of Engineering, Tohoku University, 6-6-11-404 Aoba, Aramaki, Aoba-ku, Sendai 980-8579, Japan. ^{f)}Department of Pharmacokinetics and Pharmacodynamics, School of Pharmaceutical Sciences, University of Shizuoka, 52-1 Yada, Suruga-ku, Shizuoka 422-8526, Japan. (Received November 25, 2008. Accepted December 15, 2008.)

Abstract

Shishihakuhito is a traditional Chinese medicine that is effective in the treatment of pruritic skin diseases of the elderly and atopic dermatitis. However, the molecular mechanism of such a beneficial action of shishihakuhito on the dermatologic disorders is unknown. In the present study, we for the first time examined whether shishihakuhito extract exhibits the inhibitory action on IgE-mediated histamine release from the rat basophilic leukocyte cell line, RBL-2H3 cells. The extract decreased IgE-mediated histamine release at 30 µg/ml, and more potently inhibited the histamine release at the concentrations of more than 100 µg/ml, with almost abolishing it at 200–400 µg/ml. Shishihakuhito consists of Gardenia Fruit, Phellodendron Bark and Glycyrrhiza. Next, to address the question of which crude drug components of shishihakuhito are responsible for the inhibitory action on IgE-mediated histamine release from RBL-2H3 cells, the activity of the Kampo preparations excluding each bulk extract of the shishihakuhito to inhibit the histamine release from RBL-2H3 cells was examined. The Kampo preparations without Gardenia Fruit or Glycyrrhiza at 100 µg/ml still prevented the histamine release, whereas that without Phellodendron Bark failed to inhibit the histamine release, demonstrating that Phellodendron Bark extract is mainly involved in the inhibitory action of shishihakuhito extract. In addition, shishihakuhito extract and the Kampo preparations excluding each bulk extract of the Kampo medicine inhibited lipopolysaccharide (LPS)-induced stimulation of NF-κB-dependent transcription in C6 rat glioma cells. These results suggest that shishihakuhito may exhibit the *in vivo* antagonistic action on allergic inflammation by inhibition of histamine release as well as LPS-induced stimulation of NF-κB-dependent transcription.

Key words shishihakuhito, atopic dermatitis, RBL-2H3 cells, histamine release, NF-κB-dependent transcription, C6 rat glioma cells.

#Present address: Department of Pharmacotherapy, Graduate School of Pharmacological Sciences, Tohoku University, Sendai 980-8578, Japan

*To whom correspondence should be addressed.

e-mail : yamakuni@mail.pharm.tohoku.ac.jp

Introduction

Mast cells are distributed widely throughout the body and are important in the development of acute inflammation and allergic disorders such as asthma.¹⁾ Histamine is an important chemical mediator of allergic inflammation, and is produced and stored in cytoplasmic granules in tissue mast cells and basophils.²⁾ Within seconds to minutes of activation, mast cells release histamine and other mediators, including enzymes and chemotactic factors, from their secretory granules. Released histamine increases vascular permeability and recruits inflammatory leukocytes.³⁾ Therefore, the suppression of histamine release from mast cells is a critical step in clinically regulating of allergic inflammation.

In addition to histamine, prostaglandins (PGs), arachidonic acid metabolites of the cyclooxygenase (COX) pathway, are also known to act as major mediators in the regulation of inflammation.⁴⁾ COX is the rate-limiting enzymes in the production of PGs. Inducible COX (COX-2) gene is an early inducible gene in response to many inflammatory cytokines, including IL-1,⁵⁾ TNF α ⁶⁾ and bacterial wall protein, lipopolysaccharide (LPS),⁷⁾ through nuclear factor- κ B (NF- κ B). Similarly, NF- κ B serves as a central regulator of the transcription of inflammatory cytokines, including IL-1, -2, -6, and -8, TNF α , and inducible nitric oxide synthase (iNOS).⁸⁾ Accordingly, it has been suggested that clinical control of NF- κ B-mediated transcription appears to be beneficial to patients with inflammatory conditions.

Shishihakuhito has been used as the traditional Chinese medicine for the treatment of facial eczema accompanied by inflammation from many years ago. Recently, the extract of the traditional Chinese medicine has been also revealed to be beneficial to patients with pruritic skin diseases of the elderly and atopic dermatitis.^{9,10)} Here we provide the first evidence that shishihakuhito extract inhibits IgE-mediated histamine release from rat RBL-2H3 basophilic leukocyte cells (RBL-2H3 cells), and that all of the Kampo preparations excluding each bulk extract prevent lipopolysaccharide-induced stimulation of NF- κ B-dependent transcription as potently as shishihakuhito in C6 rat glioma cells.

Materials and Methods

Materials: Shishihakuhito consists of three crude drugs: Gardenia Fruit 3.0 g, Phellodendron Bark 2.0 g, and Glycyrrhiza 1.0 g. Shishihakuhito extract and the three kinds of the Kampo preparations excluding each bulk extract of the traditional Chinese medicine employed in the present study were imported from China and kindly supplied from Kotaro Pharmaceutical Co., Ltd (Osaka, Japan). Shishihakuhito extract and other Kampo preparations excluding each bulk extract of the Kampo medicine were dissolved in dimethylsulfoxide (DMSO) and diluted with the PIPES buffer for the assays. The final concentration of DMSO used as a vehicle was adjusted to 0.2% (v/v) in all experiments.

Fetal bovine serum (FBS) for RBL-2H3 cells, FBS for C6 rat glioma cells, Eagle's minimum essential medium (MEM), and horse serum and Ham's F-10 medium were purchased from MP Biomedicals Inc. (Irvine, CA), JRH Biosciences (Lenexa, KS), Nissui Pharmaceutical (Tokyo, Japan), and GIBCO (Invitrogen, Carlsbad, CA), respectively. Anti-14-3-3 β and anti-I κ B antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). EndoFree Plasmid Maxi Kit, and phRG-TK vector and Dual-Luciferase[®] Reporter Assay System were purchased from QIAGEN K.K. (Tokyo, Japan) and Promega (Madison, WI), respectively. Dinitrophenylated-human serum albumin (DNP-HSA) was purchased from Sigma-Aldrich (St Louis, MO, U.S.A) and anti-DNP IgE-producing hybridoma were gifts from Dr. Kazutaka Maeyama (Ehime University, Ehime, Japan).

Cell culture: The RBL-2H3 cells were cultured in MEM with 15% of FBS and 0.7% of penicillin-streptomycin solution (Sigma-Aldrich, St Louis, MO, U.S.A) in a flask at 37°C in a humidified atmosphere of 5% of CO₂ in air, as described previously.¹¹⁾ C6 rat glioma cells were also grown in Ham's F-10 medium containing 15% horse serum and 2.5% fetal bovine serum in a 37°C humidified incubator in an atmosphere of 5% CO₂-95% air, as described previously.¹²⁾

Treatment of cultured cells with agents and histamine release assay: For histamine release experiment, RBL-2H3 cells were seeded in 24-well culture plates (5 x 10⁵ cells/well) and sensitized with approximately 0.2

$\mu\text{g/ml}$ of monoclonal IgE overnight. The medium was replaced with 0.18 ml of PIPES buffer (25 mM PIPES, 119 mM NaCl, 5 mM KCl, 5.6 mM glucose, 0.4 mM MgCl_2 , 1 mM CaCl_2 , 40 mM NaOH, 0.1% BSA, pH7.2), 1-400 $\mu\text{g/ml}$ of shishihakuhito extract and the same buffer including other Kampo preparations excluding each bulk extract, and then preincubated at 37 °C for 10 min. Cells were finally stimulated with 100 ng/ml of DNP-HSA as an antigen for 30 min. Released histamine in the supernatants was measured by HPLC-fluorometry as described previously.¹¹⁾

DNA transfection and luciferase assay: For transcription activity assay, C6 cells were plated into 48-well plates coated with poly-L-lysine at the density of 1.2×10^4 cells per well. After 48 h, cells were transfected with 0.19 μg of pNF- κB -Luc firefly luciferase reporter construct containing five repeated NF- κB -responsive elements provided kindly by Dr. J. Fujisawa, as previously described.¹²⁾ Cells were co-transfected with 0.01 μg of phRG-TK plasmid to normalize transfection efficiency. Thirteen h later, cells were preincubated in the presence or absence of the tested Kampo preparations including shishihakuhito extract for 3 h and were incubated with or without 1 $\mu\text{g/ml}$ LPS for 8 h. Luciferase activity was determined as previously described.^{12,13)}

Statistical analyses: Statistical analysis was performed using Student's *t*-test. A level of $p < 0.05$ was considered statistically significant.

Results and Discussion

The rat basophilic leukemia cells line, RBL-2H3, is a cultured analogue of mucosal mast cells,¹⁴⁾ where the aggregation of IgE receptors initiates the hydrolysis of inositol phospholipids, thereby increasing intracellular calcium concentration to induce histamine release.^{15,16)} Since this cell line is thus an excellent model system suitable for examining whether chemical agents, including traditional Chinese medicines, are capable of inhibiting histamine release, we first examined the effects of shishihakuhito extract on IgE-mediated histamine release from RBL-2H3 cells. The extract at 30 $\mu\text{g/ml}$ was observed to prevent significantly IgE-mediated histamine release in RBL-2H3 cells, and more potently suppressed it at the concentration of more than 100 $\mu\text{g/ml}$ (Fig. 1B), with almost completely inhibiting the histamine release at 200-400 $\mu\text{g/ml}$ (Fig. 1A). In addition, shishihakuhito extract at 400 $\mu\text{g/ml}$ appeared to increase significantly the basal level of histamine release (Fig. 1A). Furthermore, shishihakuhito extract showed few effects

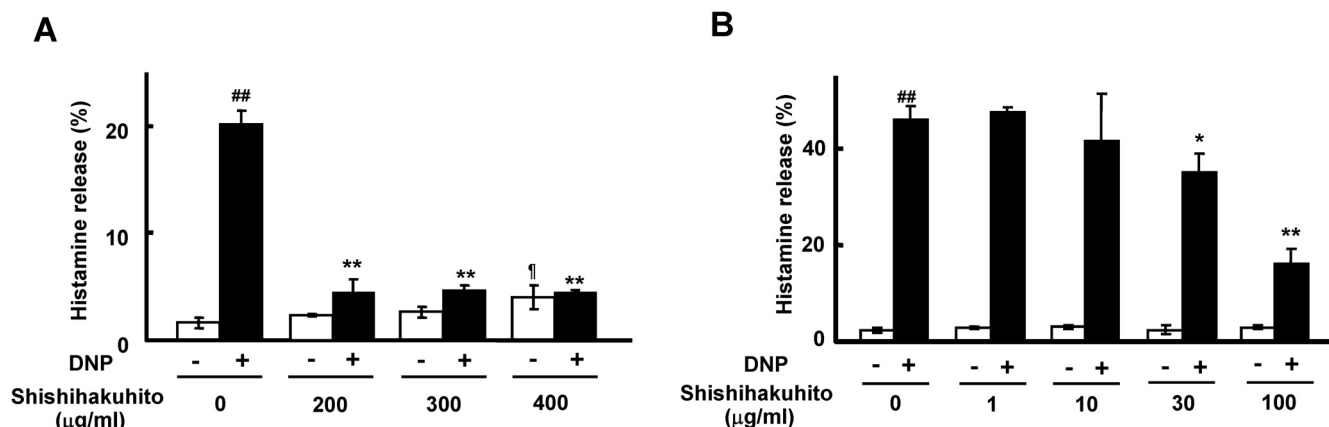


Fig. 1 Concentration-dependent inhibitory effects of shishihakuhito extract on IgE-mediated histamine release in RBL-2H3 cells.

RBL-2H3 cells were seeded in 24-well culture plates and sensitized with approximately 0.2 $\mu\text{g/ml}$ of anti-DNP IgE overnight. Thereafter, cells were preincubated for 10 min in the presence of shishihakuhito extract at the concentrations of 0, 200, 300, and 400 $\mu\text{g/ml}$ (A) or 0, 10, 30 and 100 $\mu\text{g/ml}$ (B), and then stimulated with 100 ng/ml DNP-HSA for 30 min. Histamine release was expressed as a percentage of the histamine content of cells untreated with DNP-HSA. Each column represents the mean $\pm$ S.E.M. ($n = 3$). ## $p < 0.01$ compared with cells treated without DNP-HSA and shishihakuhito extract. * $p < 0.05$, ** $p < 0.01$ compared with cells treated with DNP in the absence of shishihakuhito extract. † $p < 0.05$ compared with cells treated without DNP-HSA and shishihakuhito extract. DNP: DNP-HSA.

on the cell viability of RBL-2H3 cells, as determined by MTT assay after treatment with the extract at the concentrations ranging from 10 to 1,000 $\mu\text{g/ml}$ for 24 h (data not shown). These results thus suggest that shishihakuhito extract inhibits IgE-mediated histamine release in a concentration-dependent manner in RBL-2H3 cells, with no effects on the cell viability. Next, to examine which component of shishihakuhito are involved in the inhibitory action on IgE-mediated histamine release from RBL-2H3 cells, we assayed the activity of the Kampo preparations excluding each bulk extract of the shishihakuhito to inhibit the IgE-mediated histamine release from RBL-2H3 cells. The Kampo preparations without Gardenia Fruit or Glycyrrhiza extracts at the concentration of 100 $\mu\text{g/ml}$ still showed the activity to inhibit the IgE-mediated histamine release,

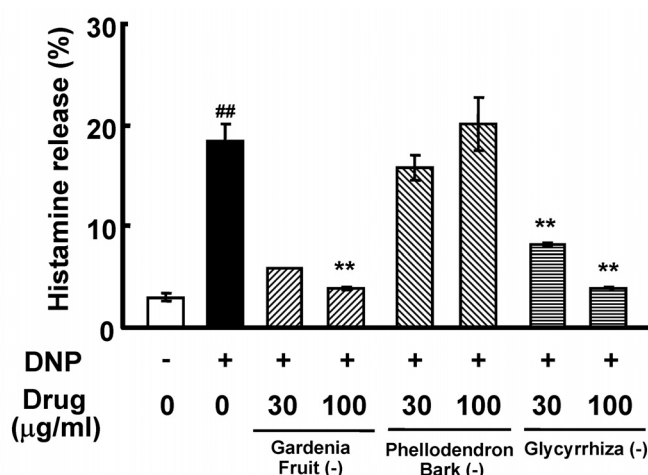


Fig. 2 Effects of shishihakuhito extract and the Kampo preparations excluding each bulk extract of the traditional Chinese medicine on IgE-mediated histamine release in RBL-2H3 cells. RBL-2H3 cells were seeded in 24-well culture plates and sensitized with approximately 0.2 $\mu\text{g/ml}$ of anti-DNP IgE overnight. Thereafter, cells were preincubated for 10 min in the presence of the Kampo preparations excluding each bulk extract of the traditional Chinese medicine at the indicated concentrations and then stimulated with 100 ng/ml DNP-HSA for 30 min. Histamine release was expressed as a percentage of histamine content of cells untreated with DNP-HSA. Each column represents the mean $\pm$ S.E.M. ($n = 3$). ^{##} $p < 0.01$ compared with cells treated without DNP-HSA and the Kampo preparations excluding each bulk extract of the traditional Chinese medicine. ^{**} $p < 0.01$ compared with cells treated with DNP-HSA in the absence of the Kampo preparations excluding each bulk extract of the traditional Chinese medicine. DNP: DNP-HSA; Gardenia Fruit (-): Shishihakuhito extract without Gardenia Fruit one; Phellodendron Bark (-): Shishihakuhito extract without Phellodendron Bark one; Glycyrrhiza (-): Shishihakuhito extract without Glycyrrhiza one.

and in contrast that without Phellodendron Bark extract failed to prevent the histamine release at the same concentration (Fig. 2), demonstrating that among three crude drug components, Phellodendron Bark is in large involved in the inhibitory action of shishihakuhito on the IgE-mediated histamine release from RBL-2H3 cells.

COX-2 gene is an early inducible gene in response to many inflammatory cytokines, including IL-1,⁵⁾ TNF α ⁶⁾ and LPS,⁷⁾ through NF- κ B activation, suggesting that clinical control of NF- κ B-mediated transcription appears beneficial to patients with inflammatory conditions. C6 rat glioma cells are well-known as an *in vitro* system suitable for studying of molecular mechanism of inflammation and have been documented to increase NF- κ B-dependent transcription in response to LPS treatment.^{12,13)} Recently, we have reported that shishihakuhito inhibits LPS-induced NF- κ B-dependent transcription in a concentration-dependent manner and prevents LPS-induced activation of COX-2 gene transcription in C6 rat glioma cells.¹⁷⁾ Therefore, to examine which crude drug components of shishihakuhito are involved in the inhibitory action of the traditional Chinese medicine on LPS-induced NF- κ B-dependent transcription activity in C6 cells, we further performed transient transfection of C6 cells with pNF κ B-Luc, an NF- κ B-dependent luciferase reporter plasmid, and then treated with vehicle, 10-200 $\mu\text{g/ml}$ of shishihakuhito extract or 50 and 100 $\mu\text{g/ml}$ of the Kampo preparations excluding each bulk extract of shishihakuhito in the absence or presence of LPS to assay the luciferase activity. Consistent with our previously reported results,¹⁷⁾ shishihakuhito inhibited LPS-induced NF- κ B-dependent transcription activity at the concentrations of more than 30 $\mu\text{g/ml}$, but without concentration-dependently inhibitory effects at the tested concentrations ranging from 30 to 200 $\mu\text{g/ml}$ (Fig. 3). Also, it was revealed that all of the tested Kampo preparations excluding each bulk extract of shishihakuhito still showed such inhibitory effects on LPS-induced NF- κ B-dependent transcription (Fig. 3), raising the possibility that each component of shishihakuhito may co-operatively inhibit LPS-induced NF- κ B-dependent transcription in C6 cells, although Phellodendron Bark alone appears to be capable of moderately preventing LPS-induced NF- κ B-dependent transcription at the concentrations of 100 $\mu\text{g/ml}$ in the

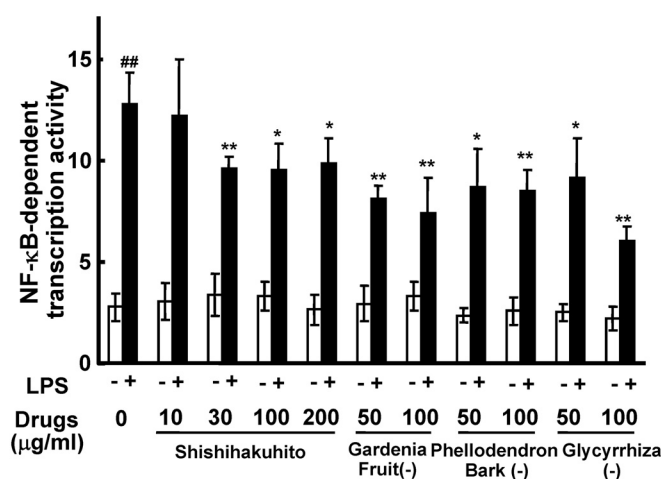


Fig. 3 Effects of shishihakuhito extract and the Kampo preparations excluding each bulk extract of traditional Chinese medicine on LPS-induced NF- κ B dependent transcription in C6 rat glioma cells.

C6 cells were plated at a density of 1.2×10^4 cells/well were cultured for 48 h. Cells were transfected with 0.19 μ g of pNF- κ B-Luc reporter plasmid and 0.01 μ g of pRG-TK plasmid for 13 h. After treatment with shishihakuhito extract and the Kampo preparations excluding each bulk extract of the traditional Chinese medicine at the indicated concentrations for 3 h, cells were stimulated with 1 μ g/ml LPS for 8 h. Cells were harvested to determine each luciferase activity. Luciferase activity was determined as described in the experimental procedures, and relative luciferase activity was calculated as the ratio of firefly luciferase activity to Renilla luciferase one. Each column represents the mean $\pm$ S.E.M ($n = 4$). ## $p < 0.01$ compared with cells treated without LPS and the Kampo preparations excluding each bulk extract of the traditional Chinese medicine. * $p < 0.01$, ** $p < 0.001$, compared with cells with LPS in the absence of shishihakuhito extract and the Kampo preparations excluding each bulk extract of the traditional Chinese medicine. Gardenia Fruit (-): Shishihakuhito extract without Gardenia Fruit one; Phellodendron Bark (-): Shishihakuhito extract without Phellodendron Bark one; Glycyrrhiza (-): Shishihakuhito extract without Glycyrrhiza one.

same cell line, as reported previously.¹⁷⁾

The most important finding of the present study is that shishihakuhito extract has indeed the pharmacological activity to prevent IgE-mediated histamine release from RBL-2H3 cells and raise the possibility that Phellodendron Bark can serve as the crude drug component which is responsible for the inhibitory action of the traditional Chinese medicine extract on the IgE-mediated histamine release from RBL-2H3 cells, suggesting that shishihakuhito may suppress histamine release from mast cells, thereby exhibiting the therapeutic action on atopic dermatitis *in vivo*.

In conclusion, to our knowledge, this study is the

first to demonstrate that the mechanism underlying the therapeutic effects of shishihakuhito on pruritic skin diseases of the elderly and atopic dermatitis may involve suppression of IgE-mediated histamine release from mast cells and that each component of the traditional Chinese medicine appears to co-operatively act to prevent LPS-induced activation of NF- κ B-dependent transcription, which controls the expression of inflammatory genes, including COX-2 gene, in C6 cells. These findings at least in part account for the preventive action of shishihakuhito on allergic inflammation *in vivo*.

Acknowledgements

We thank Dr. H. Inoue, National Cardiovascular Center Research Institute, for his helpful discussion and Dr. J. Fujisawa, Kansai Medical University, for providing an NF- κ B promoter construct.

References

- 1) Tamasky, P.R., Van Arsdel, P.P.: Antihistamine therapy in allergic rhinitis. *J. Fam. Pract.*, **30**, 71-80, 1990.
- 2) Simons, F.E.R. and Simons, K.J.: The pharmacology and use of H1-receptor antagonist drugs. *N. Engl. J. Med.*, **330**, 1663-1670, 1994.
- 3) Hart, P.H.: Regulation of the inflammatory response in asthma by mast cell products. *Immunol. Cell Biol.* **79**, 149-153, 2001.
- 4) Smith, W.L., Dewitt, D.L., Garavito, R.M.: Cyclooxygenase: structural, cellular, and molecular biology. *Annu Rev Biochem.* **69**, 145-182, 2000.
- 5) Newton, R., Kuitert, L.M., Bergmann, M., Adcock, I.M., Barnes, P.J.: Evidence for involvement of NF-kappaB in the transcriptional control of COX-2 gene expression by IL-1beta. *Biochem. Biophys. Res. Commun.*, **237**, 28-32, 1999.
- 6) Yamamoto, K., Arakawa, T., Ueda, N., Yamamoto, S.: Transcriptional roles of nuclear factor κ B and nuclear factor-interleukin-6 in the tumor necrosis factor κ -dependent induction of cyclooxygenase-2 in MC3T3-E1 cells. *J. Biol. Chem.*, **270**, 31315-31320, 1995.
- 7) Inoue, H., Yokoyama, C., Hara, S., Yoshimori, T., Tanabe, T.: Transcriptional regulation of human

- prostaglandin-endoperoxide synthase-2 gene by lipopolysaccharide and phorbol ester in vascular endothelial cells. *J. Biol. Chem.*, **270**, 24965-24971, 1995.
- 8) Baeuerle, P.A., Henkel, T.: Function and activation of NF-kappa B in the immune system. *Annu. Rev. Immunol.*, **12**, 141-179, 1994.
- 9) Toyoda, M., T., Morohashi, M.: Nerve growth factor and substance P are useful plasma markers of disease activity in atopic dermatitis. *Br. J. Dermatol.*, **147**, 71-79, 2002.
- 10) Shigeta, Y., Yonemitsu, Y., Matsumoto, R.: Clinical and experimental features of Shishihakuhito. *Pharmacometrics*, **71**, 67-72, 2006.
- 11) Maeyama, K., Taguchi, Y., Sakurai, E., Sasaki, M., Yamatodani, A., Watanabe, T.: Effects of Inhibitors of Protein Kinase C on the Release and Synthesis of Histamine in Rat Basophilic Leukemia Cells (2H3). *Jpn. J. Pharmacol.*, **58**, 291-298, 1992.
- 12) Nakatani, K., Yamakuni, T., Kondo, N., Arakawa, T., Oosawa, K., Shimura, S., Inoue, H., Ohizumi, Y.: γ -Mangostin inhibits inhibitor- κ B kinase activity and decreases lipopolysaccharide-induced cyclooxygenase-2 gene expression in C6 rat glioma cells. *Mol. Pharmacol.*, **66**, 667-674, 2004.
- 13) Yamakuni, T., Aoki, K., Nakatani, K., Kondo, N., Oku, H., Ishiguro, K., Ohizumi, Y.: Garcinone B reduces prostaglandin E2 release and NF- κ B-mediated transcription in C6 cells. *Neurosci. Lett.*, **394**, 206-210, 2006.
- 14) Seldin, D.C., Adelman, S., Austen, K.F., Stevens, R.L., Hein, A., Caulfield, J.P., Woodbury, R.G.: Homology of rat basophilic leukemia cells products and the rat mucosal mast cells. *Proc. Natl. Acad. Sci. USA.*, **82**, 3871-3875, 1985.
- 15) Beaven, M.A., Moore, J.P., Smith, G.A., Hesketh, T.R., Metcalf, J.C.: The calcium signal and phosphatidylinositol breakdown in 2 H 3 cells. *J. Biol. Chem.*, **259**, 7137-7142, 1984.
- 16) Maeyama, K., Hohman, R.J., Metzger, H., Beaven, M.A.: Quantitative relationships between aggregation of IgE receptors, generation of intracellular signals, and histamine secretion in rat basophilic leukemia (2H3) cells. Enhances responses with heavy water. *J. Biol. Chem.*, **261**, 2583- 2592, 1986.
- 17) Aoki, K., Yoshida, M., Ohizumi, Y., Yamakuni, T.: Shishihakuhito, a traditional Chinese medicine for atopic dermatitis, prevents lipopolysaccharide-induced activation of NF- κ B-dependent transcription in C6 rat glioma cells. *Pharmacometrics*, **74**, 107-110, 2008.