Short Communication

Shishihakuhito, a Kampo medicine for atopic dermatitis, suppresses NGF-induced neurite extension by inhibition of MEK/ERK signaling in PC12D cells

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Abstract

Accumulating evidence obtained from clinical studies as well as animal studies suggests that nerve growth factor (NGF) may play a crucial role in the pathogenic mechanism of atopic dermatitis (AD), involving severe scratching due to itching. Shishihakuhito is a Kampo medicine for AD. In the current study, we for the first time report that shishihakuhito extract has the activity to prevent NGF-induced stimulation of phosphorylation of MEK and ERK which has been demonstrated to mediate neurite outgrowth induced by this neurotrophic factor, and actually inhibit NGF-induced neurite extension in PC12D cells, a cellular model of peripheral sensory neurons. These findings suggest that shishihakuhito might prevent NGF signaling, thereby reducing the induction of severe itching and scratching in AD.

Key words shishihakuhito, atopic dermatitis, NGF-signaling.

Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin disease characterized by pruritic and eczematous skin lesions. Since itch-scratch cycle aggravates skin lesions in patients with AD, the control of itch is a critical step in treatment of AD. Accumulating evidence obtained from clinical studies as well as by in vivo investigations in AD model animals suggests that nerve growth factor (NGF) serves as a useful biomarker for AD and is implicated in the pathogenesis of this disease, including severe scratching due to itching. In addition, it has been reported that the expression of trkA and p75 are increased on peripheral blood eosinophils of patients with AD. Studies using atopic dermatitis models, NC/Nga or NC/NgaTnd mice, have demonstrated that NGF biosynthesis is promoted by the cells, including keratinocytes and fibroblasts, in the affected skins of these animal models, accompanied by an increment of plasma NGF level, and that protein gene product 9.5-positive peripheral sensory nerve fibers innervating to the skins extend from dermis toward the epidermis. These reported findings suggest the possible implication of NGF in the induction of severe itching in AD. In support of the notion, it has been more recently reported that the level of NGF in the horn layer is significantly higher in AD patients than in healthy...
controls, and correlates with the severity of itch.\textsuperscript{12} Shishihakuhito, a Kampo medicine, has been recently revealed to be beneficial to patients with pruritic skin diseases of the elderly and AD.\textsuperscript{13} We have earlier found that shishihakuhito not only inhibits LPS-induced NF-kB-dependent transcription in a concentration-dependent manner in C6 rat glioma cells,\textsuperscript{14} but also suppresses IgE-mediated histamine release from RBL-2H3 cells in a concentration-dependent mode.\textsuperscript{15} Here we provide the first evidence that in a rat pheochromocytoma cell line, PC12D cells which can extend neurites from the cell bodies in response to NGF like peripheral sensory neurons, shishihakuhito prevents NGF-induced increase in the phosphorylation of extracellular signal-regulated kinase (ERK) and mitogen-activated protein kinase/ERK kinase (MEK) which is required for NGF-signaling, and suppresses NGF-induced neurite extension in PC12D cells.

Materials and Methods

Materials: Shishihakuhito consists of three crude drugs: Gardenia Fruit (Gardeniae Fructus) 3.0 g, Phellodendron Bark (Phellodendri Cortex) 2.0 g, and Glycyrrhiza (Glycyrrhizae Radix) 1.0 g. Shishihakuhito extract and three kinds of the Kampo preparations excluding each bulk extract of the Kampo medicine employed in this study were manufactured and kindly supplied by Kotaro Pharmaceutical Co., Ltd (Osaka, Japan). The yield of shishihakuhito extract was 22.4\%, while those of Kampo preparation without Gardenia Fruit, the one without Phellodendron Bark and the one without Glycyrrhiza were approximately 16\%, 19\% and 21\%, respectively. Shishihakuhito extract and the other Kampo preparations were also dissolved in dimethylsulfoxide (DMSO). The final concentration of DMSO which was used as a vehicle was adjusted to 0.2\% (v/v) in all experiments. Dulbecco’s Modified Eagle’s medium (DMEM), Horse serum (HS), and fetal calf serum (FCS) were purchased from Nissui Pharmaceutical (Tokyo, Japan), GIBCO (invitogen, Carlsbad, CA), and ICN Biochemical, Inc. (Costa Mesa, CA, USA), respectively. Other chemicals and drugs were of reagent grade or of the highest quality.

Three-dimensional HPLC: Shishihakuhito was dissolved with 50\% CH\textsubscript{3}OH, filtered and analyzed by HPLC (Hitachi L-7000 series) under the following condition (Fig. 1): The sample (20 μl) was applied to a ODS column (Miglysil RP-18 GP S, 4.6 × 150 mm, 5 μm, Kanto Chemical co., INC). The elution sequence was 5 mM NaH\textsubscript{2}PO\textsubscript{4}/CH\textsubscript{3}CN (9 : 1) for the first 5 min and a linear gradient to 5 mM NaH\textsubscript{2}PO\textsubscript{4}/CH\textsubscript{3}CN (1 : 1) over 55 min. The flow rate was 0.5 ml/min and the column temperature was set at 40°C. The UV spectrum ranging from 200 to 400 nm was collected with a PDA detector (Hitachi L-7450).

Cell culture: PC12D cells were maintained at 37°C in a humidified atmosphere of 5\% CO\textsubscript{2} and 95\% air in high glucose DMEM supplemented with 5% fetal calf serum, 10% horse serum and 2 mM glutamine, as described previously.\textsuperscript{16,17}

Fig. 1 Three-dimensional HPLC chromatograms of shishihakuhito extract. Analysis of the extracts from shishihakuhito was performed by using three-dimensional HPLC according to the experimental conditions described under the Materials and Methods section.

Neurite growth assay: Assay of neurite outgrowth from PC12D cells using a phase-contrast microscopy was carried out, as described previously,\textsuperscript{16,17} following a 48-h incubation with vehicle (0.2% DMSO), shishihakuhito extract and three kinds of the Kampo preparations excluding each bulk extract of the Kampo medicine at the indicated concentrations in the presence and absence of 10 ng/ml NGF. Briefly, cells were dissociated by incubation with 1 mM EGTA in phosphate-buffered saline (PBS) for 1 h and were plated at the
density of 2 x 10^4 cells/well on 24-well culture plates coated with poly-L-lysine. After 24 h, the medium was changed to an appropriate test medium supplemented with 1% fetal calf serum and 2% horse serum containing shishihakuhito and the Kampo preparations tested. After 48 h, each culture was fixed with 2% glutaraldehyde in PBS and stored in PBS solution. Neurite outgrowth from PC12D cells was monitored under a phase-contrast microscope. Processes with lengths equivalent to one or more diameters of a cell body were scored as neurites. The effects of shishihakuhito and these Kampo preparations tested were assessed by examining the proportion of neurite-bearing cells to total cells counted. For the assay, at least 300 cells were counted in the randomly selected areas.

**Western blotting:** Cell extract preparation and Western blot analysis were conducted as described previously. In brief, after treatment with shishihakuhito and/or NGF, cells were lysed with 90 μl of lysis buffer (1 mM EDTA, 1% SDS, 10 mM NaF, 10 mM calyculin, 320 mM okadaic acid, 1 mM sodium orthovanadate, 1 mM p-APMSF, 10 μg/ml pepstatin, 10 μg/ml antipain, 10 μg/ml leupeptin, 10 μg/ml chymostatin, 10 μg/ml phosphoramidon, 10 mM HEPES, pH 7.5) per 35-mm dish. Cell lysates were heated at 95°C for 5 min, sonicated and centrifuged at 14,000 rpm for 20 min at 4°C to obtain the supernatants as cell extracts. Cell extracts were separated by 12.5% SDS-PAGE and transferred onto PVDF membrane. The blotted membrane was blocked in Tris buffered saline containing Tween-20 (TBST: 100 mM NaCl, 0.05% Tween 20, 10 mM Tris-HCl, pH 7.5) containing 5% bovine serum albumin (BSA) for 1 h at room temperature. The membrane was thereafter incubated successively with either anti-phospho-ERK (1:1000, Thr 202/Tyr 204, #9101, Cell Signaling Technology) or anti-phospho-MEK antibodies (1:1000, #9154, Cell Signaling Technology) in 5% BSA/TBST buffer overnight at 4°C, and horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (1:2000, #7074, Cell Signaling Technology) for 1 h at room temperature. Immunoreactivities were visualized with SuperSignal West Pico Chemiluminescent Substrate (Pierce). Following stripping the antibodies and the subsequent blocking with 2% and 5% BSA/TBST buffer for detection of total ERK1/2 and total MEK1/2, respectively, the membranes were reprobed with anti-total ERK (1:2500, V114A, Promega) or anti-total MEK (1:1000, #9126, Cell Signaling Technology) antibodies for overnight at 4°C, and subsequently incubated with HRP-conjugated anti-rabbit IgG for visualization of immunoreactivities as described above. The band intensities were quantitatively analyzed using SCION image software as described previously.

**Statistical analyses:** Analysis of variance (ANOVA) with post-hoc correction according to Tukey was performed on multiple comparisons. A level of p < 0.05 was considered to be statistically significant.

**Results and Discussion**

A rat pheochromocytoma cell line, PC12 cells, is widely used as a suitable cellular model for biochemical studies of neuronal differentiation, including NGF-induced neurite extension. Therefore, in the present study, action of shishihakuhito extract on NGF-induced neurite extension was investigated using a subclone of PC12 cell, PC12D cells. In this study, we first tried to examine whether shishihakuhito extract inhibits NGF-induced neurite extension in PC12D cells. Shishihakuhito extract at the concentration of 10 μg/ml inhibited neurite extension from PC12D cells as treated the cells with the Kampo medicine extract in the presence of 10 ng/ml of NGF, and it appeared to more potently inhibit the neurite extension at the concentrations of more than 25 μg/ml (Fig. 2), suggesting the concentration-dependency.

Shishihakuhito consists of Gardenia Fruit, Phellodendron Bark and Glycyrrhiza, as described above. To identify the components of shishihakuhito which are responsible for the inhibition of NGF-induced neurite extension from PC12D cells by the Kampo medicine, we next evaluated the activity of the Kampo preparations excluding each bulk extract of shishihakuhito to inhibit the NGF-induced neurite extension. Unexpectedly, as treated the cells with 50 μg/ml of the Kampo preparations excluding each bulk extract of shishihakuhito, all the Kampo preparations appeared to more potently suppress the NGF-induced neurite extension than shishihakuhito extract in PC12D cells, with the
Fig. 2 Inhibitory effects of shishihakuhito extract on NGF-induced neurite extension in PC12D cells.
PC12D cells plated at a density of $2 \times 10^5$ cell/well in 24-well plate coated with poly-L-lysine were cultured for 24 h. Cells were then treated with vehicle (PBS; open columns) or 10 ng/ml NGF (filled columns) in the presence and absence of shishihakuhito at the indicated concentrations for 48 h, and then their morphology was observed using a phase-contrast microscopy. Each value is the mean $\pm$ S.E. ($n = 4$). ***$p < 0.001$ compared with cells treated with vehicle (0.1% DMSO) in the presence of NGF. 

Fig. 3 Effects of the Kampo preparations excluding each bulk extract of shishihakuhito on NGF-induced neurite extension in PC12D cells.
PC12D cells plated at a density of $2 \times 10^5$ cell/well in 24-well plate coated with poly-L-lysine were cultured for 24 h. Cells were then treated with or without 50 µg/ml of the Kampo preparations excluding each bulk extract of the Kampo medicine in the presence (filled columns) or absence (open columns) of 10 ng/ml NGF for 48 h, and then their morphology was observed using a phase-contrast microscopy. Each value is the mean $\pm$ S.E. ($n = 3$). ***$p < 0.001$ compared with cells treated with vehicle alone. 

following rank order: the preparation without Phellodendron Bark $>$ the one without Gardenia Fruit $>$ the one without Glycyrrhiza (Fig. 3). In other word, the facts thus suggest that each of the three bulk extracts may have the antagonistic effect on their inhibitory actions each other, with the Phellodendron Bark extract having the most potent antagonistic action among them, and raise the possibility that the proper combination of the three components might exhibit beneficial, but not adverse, effects to patients with pruritic skin diseases. Accordingly, it is reasonable to interpret that these crude drug components of shishihakuhito may act in concert so that the Kampo medicine as a whole could inhibit NGF-induced neurite extension without any detrimental effects.

Since a sustained rise in ERK phosphorylation of which the reaction is catalyzed by MEK is required for NGF-induced neurite outgrowth in PC12 cells, Western blot analysis was conducted with anti-phospho-ERK and anti-phospho-MEK antibodies. As shown in Fig. 4, Western blot analysis showed that shishihakuhito extract tended to decrease the basal levels of phoshorylation of ERK and MEK in PC12D cells, but the decreasing effects appeared not to be significant. It was also shown that a 10-min treatment with 10 ng/ml of NGF profoundly increased phosphorylation of ERK and MEK in PC12D cells, whereas treatment with 50 µg/ml of shishihakuhito extract prior to NGF treatment inhibited the NGF-induced increases in phosphorylation of ERK and MEK (Fig. 4, A and B), suggesting the prevention of NGF signaling by suppression of NGF-induced activation of ERK and MEK in PC12D cells.

It is the most important finding of the present study that shishihakuhito extract has the activity to prevent NGF-induced neurite extension from PC12D cells, accompanied by inhibition of NGF-induced stimulation of phosphorylation of ERK and MEK. Although the question of which types of cells synthesizing NGF are actually responsible for aggravation of itch-scratch cycle in patients with AD remains to be resolved, clinical investigations of AD and studies using atopic dermatitis models suggest that keratinocytes, fibroblasts, mast cells and eosinophils are the candidates for NGF-producing cells localized in the lesioned skins of AD. Also, a clinical study on the beneficial effect of shishihakuhito on AD has shown that this Kampo medicine improves the itching sensation in patients with AD, accompanied by suppression of the innervation of sensory nerve fibers to the skins and the proliferation of

Shishihakuhito prevents NGF signaling
mast cells and eosinophils, with a tendency to decrease the cell number of NGF-immuno-positive keratinocytes. Accordingly, the clinical observations raise two possibilities that shishihakuhoito extract might reduce the innervation of sensory neurons’ fibers to the skins by blockage of NGF-signaling to prevent the itching sensation, and that the Kampo medicine extract might suppress the proliferation of NGF-producing cells in the lesioned skins of patients with AD in situ. In support of the former possibility, the current study showed that the Kampo medicine extract has the activity to suppress NGF-induced neurite extension in PC12D cells, a cellular model of peripheral sensory neurons, and inhibit the increased phosphorylation of MEK/ERK induced by NGF treatment as well, although the mechanism of the action of the Kampo medicine is not completely revealed.

In conclusion, this study is the first to provide the evidence that the mechanism underlying the beneficial effects of shishihakuhoito extract on a pruritic skin disease, AD, may involve prevention of NGF-signaling in the peripheral sensory nerve fibers innervating to the skins in situ. Further studies of the precise molecular mechanism of the therapeutic effects of this Kampo medicine on AD may give us a new insight into better understanding of the pathogenesis of AD.

References