Enhancement of the Nerve Growth Factor-Mediated Neurite Outgrowth from PC12D Cells by Chinese and Paraguayan Medicinal Plants

Ping Li, Kimihiro MATSUNAGA, and Yasushi OHIZUMI*

Department of Pharmaceutical Molecular Biology, Faculty of Pharmaceutical Sciences, Tohoku University, Aoba, Aramaki, Aoba-ku, Sendai 980-8578, Japan Received February 3, 1999; accepted April 5, 1999

It is very important to search for natural compounds possessing nerve growth factor (NGF)-potentiating activity. Extracts of 7 Chinese and 10 Paraguayan medicinal plants were examined for their effects on the NGF-mediated neurite outgrowth from PC12D cells to evaluate their NGF-potentiating activities. In the methanol extracts, *Gymmopteris rufa* (LINN.) BERNH, *Ruta graveolens* LINN. and *Picrorhiza scrophulariiflora* PENNELL markedly increased the proportion of neurite-bearing cells. In the case of ethyl acetate fractions, *Equisetum giganteum* LINN. produced the most powerful enhancement of the proportion of the neurite-bearing cells, and the activities were in the following decreasing order: *Equisetum giganteum* LINN., *Gymmopteris rufa* (LINN.) BERNH, *Ruta graveolens* LINN., and *Picrorhiza scrophulariiflora* PENNELL. In the water fractions, *Imperata cylindrica*, Ginseng Radix, *Gymmopteris rufa* (LINN.) BERNH, *Gochnatia polymorpha* (LESS) CAB and *Picrorhiza scrophulariiflora* PENNELL caused a weak enhancement of the proportion of PC12D cells with neurites. Of all the extracts and fractions, the methanol extract of *Picrorhiza scrophulariiflora* PENNELL induced the longest neurites in PC12D cells. In the ethyl acetate and water fractions of *Nardostachys chinensis*, long neurites were observed although only a small proportion of PC12D cells had neurites. On the other hand, in the ethyl acetate fraction of *Equisetum giganteum* LINN., while the length of the neurites was short, the proportion of neurite-bearing cells was largest among all the extracts and fractions.

Key words nerve growth factor-potentiating activity; neurite outgrowth; PC12D cell; nerve growth factor; Chinese medicinal plant; Paraguayan medicinal plant

Dementia is one of the most common health problems in the aged population in the world. It is a dehumanizing condition for the patient, whose intellectual capacity deteriorates to the extent that the performance of routine daily activities is impaired. Several forms of dementia have been distinguished, the most common being Alzheimer's disease and cerebrovascular dementia.^{1,2)} To combat this, nerve growth factor (NGF) has held the promise of therapeutic efficacy in the treatment of neurodegenerative diseases, since NGF stimulates the outgrowth of neurites in neuronal cells and plays an important role in the survival and maintenance of neurons in the central nervous system.^{3,4)} However, NGF can be used for medical treatment only when directly injected into the brain, since it is a large molecular weight polypeptide, does not cross the blood-brain barrier and is easily metabolized by peptidases when administered peripherally. Recently, we found a novel low molecular weight neurotrophic factor from human astrocytoma cells.⁵⁾ It is very important to search for natural compounds which may become lead compounds for the development of medical drugs to treat such a serious disease as dementia.^{1,6-9)}

In our screening program for pharmacologically active substances from natural resources, we have devoted our attention to the existence of medicinal plants and crude drugs possessing NGF-potentiating activity, since these are expected to be potentially useful for the medical treatment of dementia.¹⁾ Furthermore, detailed studies of the active constituents in these plants and crude drugs are of great value for generating new types of drugs aimed at the medical treatment of dementia. In this study, we examined Chinese and Paraguayan medicinal plants which have traditionally been used mainly for sedative, diuretic and antipyretic purposes, on the NGF-mediated neurite outgrowth from PC12D cells, and found several plants with powerful NGF-potentiating ac-

tivity.

MATERIALS AND METHODS

Materials 7S NGF and poly-L-lysine were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Dulbecco's modified Eagle's medium (DMEM; high glucose) was from Gibco RBL (Grand Island, NY, U.S.A.). Fetal calf serum (FCS) and horse serum (HS) were from Cell Culture Laboratory (Cleveland, OH, U.S.A.) and from ICN Biochemical, Inc. (Costa Mesa, CA, U.S.A.), respectively. Glutaraldehyde was purchased from Wako Pure Chemical (Tokyo, Japan). All other reagents or drugs were of analytical grade. Chinese and Paraguayan medicinal plants were obtained from Matsuura Yakugyo Co., Ltd. (Nagoya, Japan) and from NKK Corporation (Tokyo, Japan), respectively.

Preparation of Extracts and Partitioned Fractions The extracts and fractions were prepared as follows (Chart 1). 17 medicinal plants were extracted with methanol at room temperature for 24 h, and the solvent was evaporated under reduced pressure to give methanol extracts. The methanol extract was then partitioned between ethyl acetate and water. Each fraction was concentrated under reduced pressure. The extracts and fractions were dissolved in dimethyl sulfoxide (DMSO). For all these experiments, the extracts and fractions

Chart 1. Preparation of Extracts and Fractions

* To whom correspondence should be addressed.

Table 1. Effects of Methanol Extracts of Medicinal Plants and Their Partitioned Fractions on the NGF-Mediated Neurite Outgrowth from PC12D Cells

Plants	Neurite-bearing cells		
	Methanol extract	Ethyl acetate fraction	Water fraction
1. <i>Nardostachys chinensis</i>	—	+	+
2. <i>Imperata cylindrica</i>	±	+	+
3. <i>Nandina domestica</i> THUNBERG	±	±	—
4. Ginseng Radix	—	—	+
5. <i>Zizyphi Fructus</i>	±	nt	—
6. <i>Acanthospermum australe</i> O.K.	—	—	—
7. <i>Zea mays</i> L.	+	+	—
8. <i>Piper fulvescens</i> C. DC.	+	—	—
9. <i>Equisetum giganteum</i> LINN.	+	+++	—
10. <i>Mentha</i> sp.	—	—	—
11. <i>Gymnopteris rufa</i> (LINN.) BERNH	++	++	+
12. <i>Gochnatia polymorpha</i> (LESS) CAB	—	—	+
13. <i>Ruta graveolens</i> LINN.	++	++	—
14. <i>Rosa blanksiae</i> AIT	—	+	—
15. <i>Baccharis gaudichaudiana</i> DC.	—	—	—
16. <i>Picrohiza scrophulariiflora</i> PENNELL	++	++	+
17. <i>Atractylodes chinensis</i> KOIDZ.	+	—	—

All extracts and fractions of medicinal plants at the concentration of 30 µg/ml were administered. +++, strongly enhanced; ++, fairly enhanced; +, enhanced; ±, weakly enhanced; —, no effect; nt, not tested.

Table 2. Effects of Methanol Extracts and Their Partitioned Fractions of 4 Medicinal Plants on the NGF-Mediated Neurite Outgrowth from PC12D Cells

Plant	Proportion of neurite-bearing cells (% of control)		
	Methanol extract	Ethyl acetate fraction	Water fraction
<i>Equisetum giganteum</i> LINN.	38.3±2.3*	101±3.8**	—
<i>Gymnopteris rufa</i> (LINN.) BERNH	73.3±5.8**	81.6±1.0**	38.3±6.2
<i>Ruta graveolens</i> LINN.	76.7±3.3**	61.7±4.7**	—
<i>Picrohiza scrophulariiflora</i> PENNELL	71.1±2.8**	52.0±5.1**	28.3±4.4

Each value represents the mean±S.E.M. (n=4). All extracts and fractions of medicinal plants at the concentration of 30 µg/ml were administered. —, no effect. Proportion of neurite-bearing cells was expressed as a percentage against the control with 30 ng/ml NGF in the absence of extracts and fractions. Significant difference from the 2 ng/ml NGF (21.5±4.4) in the absence of extracts and fractions, *p<0.05, **p<0.01.

in DMSO or 0.1% DMSO were added to the medium containing NGF (2 ng/ml), and their facilitating activity of NGF action was estimated. DMSO, at a concentration of 0.1%, had no effect on the cells.

Cell Culture and Neurite Measurement PC12D cells were cultured at 37 °C in DMEM supplemented with 5% FCS and 10% HS under a water-saturated atmosphere of 95% air and 5% CO₂, as previously reported.^{5,10} The cells were seeded in 24-well culture plates (2×10⁴ cells/well) coated with poly-L-lysine.¹⁰ After 24 h, the medium was changed to an appropriate test medium containing 2 ng/ml NGF, 1% FCS and 2% HS. After 48 h, each culture was fixed with 1% glutaraldehyde in phosphate-buffered saline (PBS) and stored in PBS. The outgrowth of neurites from PC12D cells was monitored under a phase-contrast microscope. Processes with lengths equivalent to one or more diameters of the cell body were scored as neurites. A minimum of 100 cells for the measurement of proportion of neurite-bearing cells and 10 cells for the measurement of neurite length were examined for each data point.

Statistical Analysis The results were assessed by Student's *t*-test.

RESULTS AND DISCUSSION

The effects of methanol extracts of medicinal plants and

their partitioned fractions on the NGF-mediated neurite outgrowth from PC12D cells are shown in Table 1. All extracts and partitioned fractions were administered at the concentration of 30 µg/ml. In the methanol extracts, *Gymnopteris rufa* (LINN.) BERNH, *Ruta graveolens* LINN. and *Picrohiza scrophulariiflora* PENNELL showed marked potentiating activities of neurite outgrowth from PC12D cells. *Zea mays* L., *Piper fulvescens* C. DC., *Equisetum giganteum* LINN. and *Atractylodes chinensis* KOIDZ showed fair enhancing activity. In the ethyl acetate fractions of methanol extracts, *Equisetum giganteum* LINN. (30 µg/ml) was the most powerful of the tested samples. *Gymnopteris rufa* (LINN.) BERNH, *Ruta graveolens* LINN. and *Picrohiza scrophulariiflora* PENNELL markedly enhanced the neurite outgrowth from PC12D cells. In the water fractions of methanol extracts, *Nardostachys chinensis*, *Imperata cylindrica*, Ginseng Radix, *Gymnopteris rufa* (LINN.) BERNH, *Gochnatia polymorpha* (LESS) CAB and *Picrohiza scrophulariiflora* PENNELL produced only a slight increase in the number of neurite-bearing cells. Other fractions apparently had no effect on neurite outgrowth. The pharmacological actions of *Equisetum giganteum* LINN., *Gymnopteris rufa* (LINN.) BERNH, *Ruta graveolens* LINN. and *Picrohiza scrophulariiflora* PENNELL were examined in greater detail (Table 2). The proportion of neurite-bearing cells induced by the ethyl acetate fraction of *Equisetum giganteum* LINN. was demonstrated to be comparable to that in-

duced by 30 ng/ml NGF. In the ethyl acetate fractions, the activities were in the following decreasing order: *Equisetum giganteum* LINN., *Gymnopteris rufa* (LINN.) BERNH, *Ruta graveolens* LINN., and *Picrorhiza scrophulariiflora* PENNELL. Measurement of neurite length is shown in Figs. 1 and 2. The methanol extract of *Picrorhiza scrophulariiflora* PENNELL induced the longest neurites in PC12D cells (Figs. 1 and 2c). In the ethyl acetate and water fractions of *Nardostachys chinensis*, the length of the neurites was long (Figs. 1, 2d and 2e), although the proportion of neurite-bearing cells was small

Fig. 1. Neurite Length of PC12D Cells Cultured in the Presence of Extracts and Fractions of Various Medicinal Plants

The extracts and fractions in DMSO or 0.1% DMSO were added to a medium containing NGF (2 ng/ml) and their facilitating activity of NGF action was estimated. Of the cells expressing neurites under the different medium conditions, the length of the neurites was measured (in μm). A minimum of 10 cells was examined for each data point. Lane 1, control; lane 2, 2 ng/ml NGF; lanes 3–5, *Nardostachys chinensis*; lanes 6–8, *Equisetum giganteum* LINN.; lanes 9–11, *Gymnopteris rufa* (LINN.) BERNH; lanes 12–14, *Ruta graveolens* LINN.; lanes 15–17, *Picrorhiza scrophulariiflora* PENNELL. The final concentrations of extracts and fractions were 30 $\mu\text{g}/\text{ml}$. Each value represents the mean $\pm$ S.E.M. ($n=10$). Significant difference from the 2 ng/ml NGF is indicated in the figure, * $p<0.05$, ** $p<0.01$.

(Table 1). On the other hand, in the ethyl acetate fraction of *Equisetum giganteum* LINN., the length of the neurites was short (Figs. 1 and 2f), whereas the proportion of neurite-bearing cells was large (Table 1). The ethyl acetate fractions induced longer neurites than the water fractions (Fig. 1). It is probable that effective components in the extracts and fractions were more soluble in a hydrophobic solvent. Extracts and fractions alone did not cause the neurite elongation from PC12D cells, but significantly increased the proportion of cells with neurites in cultures treated with NGF, suggesting that these extracts and fractions did not increase NGF-release from cells but facilitated a step of an NGF-receptor mediated intracellular signaling pathway.

Since bioactive compounds possessing potentiating activity for the NGF-action on neurite outgrowth from neuronal cells might be potentially beneficial to the medical treatment of dementia,¹⁾ it is profoundly interesting that some plant extracts examined in this experiment showed this activity. Active components in these plant extracts will be isolated, investigated in terms of their efficacy on animal models of dementia, and applied to the development of lead compounds for medical drugs to treat neurodegenerative disorders.

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Fig. 2. Microscopic Observation of the Neurites

a, control; b, 2 ng/ml NGF; c, the methanol extract of *Picrorhiza scrophulariiflora* PENNELL; d, the ethyl acetate fraction of *Nardostachys chinensis*; e, the water fraction of *Nardostachys chinensis*; f, the ethyl acetate fraction of *Equisetum giganteum* LINN. Scale bar = 100 μm .

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