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Neurotrophic and Neuritogenic Effects of Water Extracts of Rhizoma of *Coptis chinensis* Franch in PC12 Cells

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***Coptis chinensis* (CC) is one of the traditional herbs used in Oriental medicine for the treatment of gastrointestinal disorders, anxiety, and insomnia. In this study, neurotrophic and neuritogenic effects of CC on rat pheochromocytoma (PC12) cells were evaluated. Pretreatment of PC12 cells with water extracts of CC (120 μg /ml) produced considerable outgrowth of neurites that is comparable to the effect of nerve growth factor (NGF). Therefore, neurite outgrowth was quantified and expression of NGF mRNA was examined. Furthermore, characteristics of neurites were immunocytochemically confirmed using axon and dendrite-specific antibodies. These results suggest that water extracts of CC contain components that have neurotrophic and neuritogenic properties.**

Key Words: *Coptis chinensis*, Neurite outgrowth, PC12 cells

INTRODUCTION

Neurotrophic factors consist of a family of polypeptide growth factors that direct neuronal differentiation and survival. Over the past 50 years, several neurotrophins have been discovered and identified: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophins 3, 4/5 and 6 (NT-3, NT4/5, and NT-6). Recently, many studies have also demonstrated significant neurotrophic and neuroprotective effects of several endogenous molecules, including insulin-like growth factor 1 (IGF-1), glial-derived neurotrophic factor (GDNF), transforming growth factor (TGF), fibroblast growth factor (FGF), and cytokines such as ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), and oncostatin-M, cardiotrophin (Tuszynski & Gage, 1994; Bothwell, 1995). Many neurological disorders are often due to loss of normal neuronal function and neuronal cell death, which may accompany gradual loss of neurotrophic supports. In this regard, neurotrophic ligands or any pharmacological intervention that sustain neurotrophic actions in the brain would be expected to benefit the treatment of neurodegenerative diseases.

Recently, some Oriental medicinal herbs such as *Panax ginseng*, *Panax notoginseng*, *Gastrodia elata*, and *Coptis chinensis* (CC) are shown to have a cognitive-enhancing property in a passive avoidance test in scopolamine-induced amnesic rats (Hsieh et al, 2000). Several herb formulations including CC are proposed to be useful for the treatment of mental disorders such as depression and Alzheimer's disease (Kong et al, 2001). CC is an important ingredient of Oriental medicine that has been effectively used as a crude drug for treatment of vomiting, abdominal pain, diarrhea, and contusions (Huang, 2000). Furthermore, CC has been shown to have a strong antibacterial action on staphylococci and streptococci (Kowalewski et al, 1972; Higaki et al, 1995; Hu et al, 2000), antifungal (Nakamoto et al, 1990) and antidiarrheal action (Kase et al, 1999), anti-inflammatory effect (Yasukawa et al, 1991; Ivanovska & Philipov, 1996), antioxidant activity as a free radical scavenger (Liu & Ng, 2000; Schinella et al, 2002) as well as anti-cancer activity (Iizuka et al, 2000). Recently, Shigeta et al (2002) reported the ability of Coptidis Rhizoma extract in potentiation of NGF-induced neurite outgrowth. The main objectives of the present study are to determine whether water extracts of CC have neurotrophic and neuritogenic actions on PC12 cells.

METHODS

Materials

Dried rhizoma of CC was purchased from an Oriental herb market in Busan, South Korea. The CC (20 g) was pulverized and extracted twice in deionized water (2.5 L) at 110°C, and the extract was then filtered and evaporated to dryness under reduced pressure. The final yield of the concentrated dried residue was 5.2 g. Concentration of CC extract was measured with a refractometer using sucrose solution as a standard. NGF was purchased from Promega (USA), and antibodies to MAP-2 (SMI 52) and pan axonal neurofilament marker (SMI 312) were from Sternberger monoclonals (USA). TRI reagent (Molecular Research Center Inc., USA) was used for RNA preparation.

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ABBREVIATIONS: CC, *coptis chinensis*; PC12 cells, rat pheochromocytoma cells; NGF, nerve growth factor.

Cell culture

PC12 cells were obtained from Seoul National University Cell Line Bank (Seoul, South Korea), and were cultured as follows. The culture medium used was RPMI 1640 medium (Sigma, USA) supplemented with 10% fetal bovine serum (FBS), 100 units/ml of penicillin, and 100 μg/ml of streptomycin in a humidified atmosphere of 95% air, 5% CO_2 at 37°C. The cells were seeded onto 100 mm culture dishes at a density of 5×10^5 cells/ml to examine NGF mRNA expression. For immunocytochemistry, cells were seeded onto sterilized glass cover-slips in 6 well plates, at a density of 1×10^4 cells/ml. The cells were treated with water extracts of CC (120 μg/ml) for up to 30 days, and the medium was changed 3 times per week. For a positive control, cells were grown in the absence or presence of NGF (50 ng/ml; Promega, USA) for 14 days.

Neurite outgrowth analysis

Morphological differentiation of PC12 cells was examined from the second day of the CC treatment. The cellular response to NGF or CC water extracts was estimated by counting the number of cells showing neuritic processes under a phase-contrast microscope. The differentiation of PC12 cells was scored as follows: Photographs were taken from two representative non-overlapping regions of a culture plate, and 200 cells were counted per each dish. Spherical cells with no neurite outgrowth were given a score of 0 (S0), and they were completely undifferentiated. Cells that were elongated with minimal neurite outgrowth were scored 1 (S1). Cells that had more than two neurites and their length was less than the diameter of the cell body were given a score of 2 (S2). Cells with one or two neurites whose length was more than two times greater than the diameter of the cells were given a score of 3 (S3). Cells with more than two neurites whose length was more than double the diameter of the cells were given a score of 4 (S4), and they were considered to be maximally differentiated. The neurite index was calculated as follows: Neurite index = total neurite score ($\sum$S)/total cell number ($\sum$N). $\sum S = (1N_1) + (2N_2) + (3N_3) + (4N_4)$, where Ns equals the number of cells which have been allocated the score S (0-4) from each field (Obara et al, 1999; Rudakewich et al, 2001). All data are presented as mean ± SE of the values of five wells. The significance of differences among groups was determined by ANOVA test.

Reverse transcription-polymerase chain reaction (RT-PCR)

The total RNA from PC12 cells was extracted by using TRI reagent. The integrity of RNA was checked by formaldehyde/agarose gel electrophoresis. Total RNA of each sample was reverse transcribed into cDNAs, which in turn PCR was carried out using following primers. The sense primer (5'-CTTCAGCATTCCCTTGACAC-3', 191-210 of human NGF cDNA) and the antisense primer (5'-CGCCTTGACA

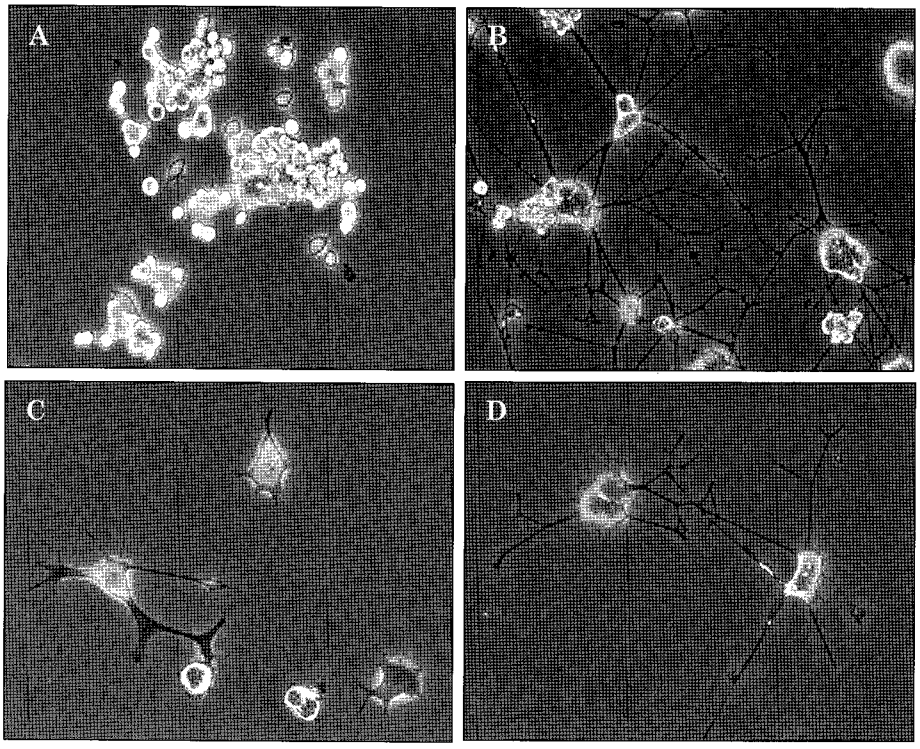

Fig. 1. Representative microphotographs, showing the neuritogenic effect of *Coptis chinensis* (CC, 120 μg/ml) water extracts. Data represent PC12 cells cultured in the presence of water extracts of CC for 4 days (C) and 14 days (D). PC12 cells cultured with 50 ng/ml of NGF for 14 days (B) were used as a positive control. Panel A represents undifferentiated PC12 cells cultured for the same period. Photographs were prepared with 200X magnification.

AAGGTGTGAGTC-3', 682-703) were complementary to conserved regions of the cDNA from mouse and human NGF. The NGF cDNA fragment was amplified 30 cycles (94°C for 30 s, 58°C for 30 s and 72°C for 30 s). The number of cycles that yielded a quantitative amount of product was determined in a preliminary experiment. The PCR products were 512 bps in size. Both reverse transcription and PCR amplification were performed in the Perkin-Elmer GeneAmp PCR system 2400. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcripts were used as a positive control. PCR products, which were separated by electrophoresis on 1.5% agarose gels and stained with ethidium bromide, were analyzed with an image scanner (Gel doc 2000, Biorad, USA).

Immunocytochemistry

For immunocytochemistry, PC12 cells were cultured onto sterilized glass cover-slips and were fixed with 4% paraformaldehyde for 30 min at room temperature. The cells were washed three times in 0.05 M Tris-HCl buffer (pH 7.6) containing 1.5% sodium chloride (1.5 T) and were then treated with 3% H_2O_2 in methanol for 30 min to reduce endogenous peroxidase. Then, the cells were washed thoroughly in 1.5 T and Tris-buffered saline containing 0.1% Triton X-100 (TBST buffer), incubated with appropriate blocking solution for 30 min to block nonspecific secondary antibody staining, and were subsequently labeled with a 1 : 2500 dilution of Pan-axonal neurofilament marker (SMI 312, Sternberger Monoclonals Inc., USA) or a 1 : 2500 dilution of monoclonal MAP 2 (SMI 52, Sternberger Monoclonals Inc., USA) at 4°C. Immunolabeling was detected by an immunoperoxidase reagent kit (The One Kit, Sternberger Monoclonals Inc., USA), and bound antibodies were visualized under a microscope (Olympus BX-50, Japan).

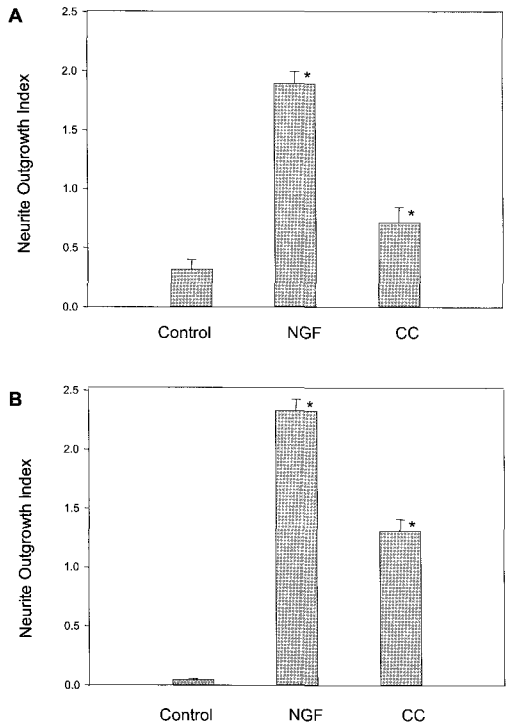

Fig. 2. Effects of *Coptis chinensis* (CC, 120 μg/ml) and NGF (50 ng/ml) on neurite outgrowth of PC12 cells. Cells were treated with either CC or NGF for 4 (A) and 14 days (B). Neurite outgrowth index refers to the extent of differentiation characteristic of the cells, with 0 being the least outgrowth, and 4 being the most extensive outgrowth. An average was calculated with about 200 cells for each treatment, and data presented are the average of triplicate samples. *indicates significantly different from non-treated control, where $P<0.05$. Data are presented as the mean ± SE.

RESULTS

Effect of Coptis chinensis on the neurite outgrowth of PC12 cells

To assess neuritogenic property of CC water extracts, PC12 cells were incubated for 4, 10, and 30 days in RPMI 1640 medium containing 10% FBS with 120 μg/ml of CC, by changing the medium every 2~3 days. For a positive control, cells were grown in the absence or presence of NGF (50 ng/ml). As presented in Fig. 1, a slight neurite sprouting was observed within 4 days of CC treatment (Fig. 1C), a significant neurite outgrowth was detected at 14 days of the treatment (Fig. 1D), and this neuritogenic effect was observed up to 30 days (Fig. 3). The neurite indices, the degree of neurite outgrowth assessed by "differentiation index" as described above, at 4 days (Fig. 2A) and 14 days (Fig. 2B) of the CC treatment demonstrated that the water extracts of CC promoted neurite outgrowth comparable to the effect of NGF (Fig. 2). This neuritogenic effect of CC was increased in a time-dependent manner (Fig. 3). Furthermore, the extracts induced the NGF mRNA expression, and the expression pattern was also time-dependent (Fig. 4).

Characterization of Coptis chinensis-induced neurite outgrowth of PC12 cells

The CC-induced neurite outgrowth was further confirmed by the immunocytological staining using axon-specific [SMI 312 (pan-axonal neurofilament marker)] and dendrite-

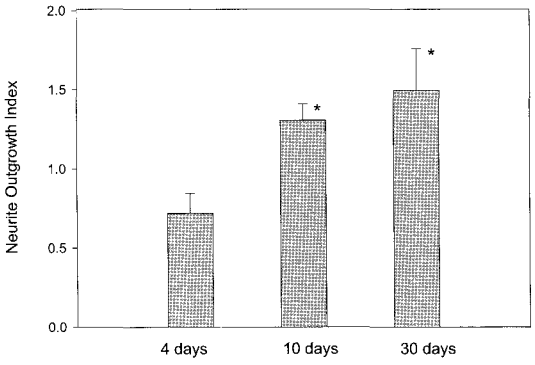

Fig. 3. Neurite outgrowth of PC12 cells by water extracts of *Coptis chinensis* (CC, 120 μg/ml), showing a time-dependent differentiation pattern (4, 10, and 30 days). Cells were treated with CC water extracts for up to 30 days. Neurite outgrowth index refers to the extent of differentiation characteristic of the cells, as described in Fig. 2.

specific [monoclonal MAP-2 (SMI 52)] antibodies, showing that the water extracts of CC were able to promote both axonal and dendritic processes (Fig. 5).

DISCUSSION

In response to NGF, PC12 cells extend neurites and develop characteristics of sympathomimetic neurons (Greene & Tischler, 1976). Therefore, PC12 cells have been used as an in vitro model of neurite differentiation. In the present study, we showed that CC induced neurite outgrowth in PC12 cells (Fig. 1), and this effect might be associated with NGF, as shown by increased expression of NGF mRNA (Fig. 4). We also demonstrated that CC induced the growth of both axon and dendrite (Fig. 5). The appearance of CC-induced neurite outgrowth was very similar to that of the NGF treatment (Fig. 1). Furthermore, the CC-induced neuritogenic effect showed a sustained neurite outgrowth up to 30 days (Fig. 3), whereas NGF did not maintain a fully differentiated state after 14 days in culture.

Axonal outgrowth is a key event in the development and function of nervous system. Recently, it has become clear that multiple extracellular and intracellular signals contribute to promote the extension of axons (Mueller, 1999; Kaplan & Miller, 2000; Sofroniew et al, 2001). Neuronal axonal growth involves precisely controlled mechanisms such as specific gene expressions, enzymes responsible for manufacturing materials for elongation of neurites, and assembly and disassembly of cytoskeletal elements, thus, requiring the total molecular program.

Many small molecules with NGF inducing activity have already been reported, mainly in astrocytes. They are structurally and functionally diverse molecules, such as active metabolite of vitamin D (Musiol & Feldman, 1997; Veenstra et al, 1998), adenosine (Heese et al, 1997), benzoquinones (Takeuchi et al, 1990), fellutamides (Yamaguchi et al, 1993b), pyrroloquinoline quinines (Yamaguchi et al, 1993a), and scabronines (Obara et al, 1999). Furthermore, there are several factors such as basic fibroblast growth factor (bFGF) and cyclic AMP (cAMP) known to induce differentiation and extension of neurite processes in PC12 cells (Pollock et al, 1990; Raffioni & Bradshaw, 1992). The neurite outgrowth in PC12 cells has been suggested to underlie several signaling mechanisms. Indeed, neurite outgrowth and extension by NGF in PC12 cells have been thought to involve mitogen-activated protein kinase (MAPK) in a central role (Cowley et al, 1994; Harada et al, 2001). MAPK activation is mediated through the calmodulin (CaM)/protein kinase A (PKA)/Rap-1/B-Raf pathway by $[Ca^{2+}]$ elevation, however, the mechanism of down-stream of MAPK remains unclear. Protein kinase C (PKC) is also involved in neurite outgrowth in response to NGF in PC12 cells, which is mediated through MAPK phosphorylation (Brodie et al, 1999). Recently, Obara et al (2000) reported that weak inhibition of DNA polymerases α and β activity induces the neurite outgrowth in PC12 cells, however, the exact molecular signaling pathways underlying neurite outgrowth in PC12 cells remain to be elucidated.

Since NGF mRNA expression was significantly increased and outgrowth of axon and dendrite of PC12 cells were shown by the treatment with CC, it is highly likely that NGF plays a direct or indirect role in neurite outgrowth. Further study is in need to clarify detailed mechanism and active components of the CC which are responsible for the

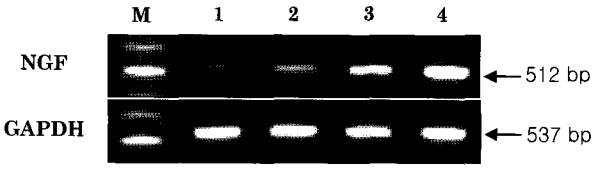

Fig. 4. Effects of *Coptis chinensis* on NGF mRNA expression in PC12 cells. The cells were harvested after 14-day treatment with either CC (120 μg/ml) or NGF (50 ng/ml). Total RNA was reverse transcribed, followed by the amplification of NGF transcripts by the RT-PCR method. Lane M, molecular weight marker (100 bp DNA ladder, Promega, USA); Lane 1, undifferentiated PC12 cells; lane 2, PC12 cells treated with CC for 4 days; lane 3, PC12 cells treated with CC for 14 days; lane 4, PC12 cells treated with NGF for 14 days. Data presented are representative of three separate experiments.

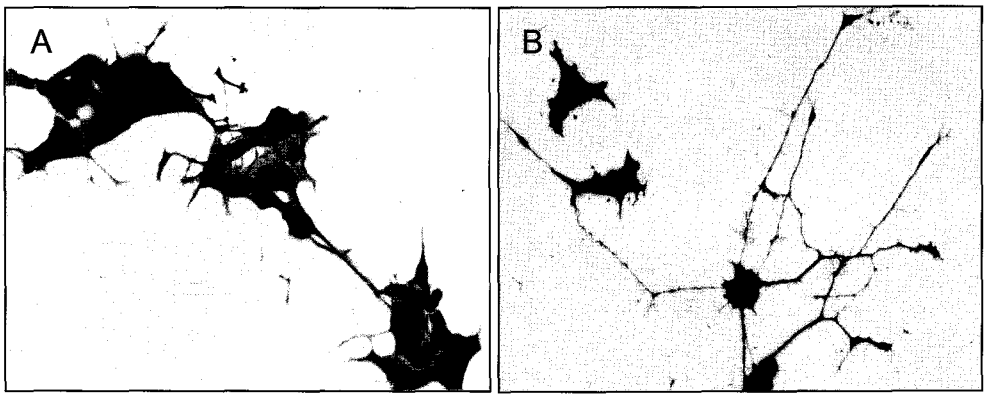

Fig. 5. Representative microphotographs, showing the immunoreactivity of (A) axon specific (pan-axonal neurofilament marker, SMI 312) and (B) dendrite specific (MAP-2, SMI 52) antibodies. PC12 cells were cultured in the presence of *Coptis chinensis* (CC, 120 μg/ml) water extracts for 14 days and immunolabeled with molecular markers of neurite development. Original magnification of the images was 40×.

neurotrophic and neuritogenic properties on PC12 cells. In conclusion, in the present study, we observed that CC has neurotrophic and neuritogenic actions in neuronal cells, and that the CC-induced neurite outgrowth is comparable to the effect of NGF in PC12 cells. This new finding might provide a logical basis for future application of CC as a potential therapeutic intervention in the treatment of many neurodegenerative disorders.

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