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Stimulation of neurotrophic factor secretion from 1321N1 human astrocytoma cells by novel diterpenoids, scabronines A and G

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Abstract

Glial cells release neurotrophic factors that maintain neurons functionally. When rat pheochromocytoma cells (PC-12) were cultivated with the conditioned medium of human astrocytoma cells (1321N1) incubated with the new diterpenoids, scabronines A and G, isolated from *Sarcodon scabrosus*, they changed their morphology and there was neurite outgrowth. The scabronines increased the expression of mRNA for nerve growth factor (NGF), and the secretion of NGF from 1321N1 cells in a concentration-dependent manner. However, the enhanced neurite outgrowth produced by the conditioned media was slightly inhibited by NGF neutralizing antibody, and the concentration of NGF released in response to the scabronines was insufficient to cause differentiation. These results suggest that scabronines cause the secretion of other factors together with NGF from 1321N1 cells. The diterpenoids are useful drugs to clarify the mechanism of synthesis and secretion of neurotrophic factors.

Keywords: 1321N1 human astrocytoma cell; PC-12 cell; Scabronine; NGF (nerve growth factor); Neurotrophic factor; Neurite outgrowth

1. Introduction

Neurotrophic factors are essential to maintain and organize neurons functionally because neurons cannot proliferate and regenerate as terminally differentiated cells. Glial cells support neurons by releasing neurotrophic factors, such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (Friedman et al., 1988; Lu et al., 1991; Rudge et al., 1995; Rush et al., 1997) and glia-derived neurotrophic factor (GDNF) (Lin et al., 1993). NGF, a neurotrophic factor, has been extensively investigated. It has pleiotrophic effects such as the induction of neuronal differentiation, the promotion of neuronal survival and the prevention of apoptosis in neurons of both central and peripheral origin (Levi-Montalcini, 1987). It has been shown that NGF synthesis in glial cells is amplified following the stimulation of β-adrenoceptors, an effect mediated via cyclic AMP (Mocchetti et al., 1989). However, the detailed mechanism of NGF synthesis still remains unknown.

Rat pheochromocytoma cells (PC-12) have been used as an in vitro model of neuronal differentiation. In response to NGF, these cells differentiate to extend neurites and to develop the characteristics of sympathetic neurons (Greene and Tischler, 1976). Human astrocytoma cells (1321N1) have been used as a model of glial cells to investigate receptor-mediated events (Nakahata and Harden, 1987; Nakahata et al., 1989, 1991). Recently, we found that 1321N1 cells released a new neurotrophic factor that causes the differentiation of PC-12 cells (Obara et al., 1998).

Sarcodon scabrosus, a bitter mushroom, contains compounds with a unique chemical structure, such as the

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sarcodonins A–H (Shibata et al., 1989). Recently, we isolated new cyathane diterpenoids from *S. scabrosus*, termed scabronines (Ohta et al., 1988).

The present study was undertaken to examine the effects of the diterpenoids scabronines A and G on the release of neurotrophic factors from 1321N1 cells.

2. Materials and methods

2.1. Materials

NGF was obtained from Sigma (St. Louis, MO, USA). The NGF enzyme-linked immunosorbent assay (ELISA) kit and the mouse neutralizing antibody against NGF were obtained from Boehringer Mannheim (Mannheim, Germany). The total RNA extraction kit used was from Pharmacia Biotech (Piscataway, NY, USA). The reverse transcription polymerase chain reaction (RT-PCR) kit was from Toyobo, (Osaka, Japan). 3,(4,5-dimethylthiazol-2-yl-) diphenyltetrazolium bromide (MTT) was from Dojindo (Kumamoto, Japan). PD98059 was from New England Biolabs, (Beverly, MA, USA).

2.2. Purification of scabronines A and G

Scabronine A was purified from the fruit bodies of the mushroom *S. scabrosus* as described previously (Ohta et al., 1988). Briefly, fruit bodies (1.4 kg) of *S. scabrosus* were extracted with methanol and then the extract was partitioned between *n*-hexane-ethyl acetate and water. The *n*-hexane-ethyl acetate extract was subjected repeatedly to silica gel chromatography, using *n*-hexane-ethyl acetate or a chloroform–methanol mixed solvent system of increasing polarity. The fraction obtained with chloroform–methanol (30:1) elution was purified twice by reversed phase high-performance liquid chromatography on a column of octadecanoyl silica gel. Elution with methanol–H_2O gave scabronine A. Scabronine G was purified in a similar way to scabronine A.

2.3. Cell culture

1321N1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum (Cell Culture Laboratory, Cleveland, OH, USA), penicillin (50 unit/ml) and streptomycin (50 μg/ml) in an incubator containing 5% CO_2 at 37°C. PC-12 cells were grown in DMEM supplemented with 10% fetal calf serum, 5% horse serum (ICN Biochemicals, Costa Mesa, CA, USA) and the antibiotics described above in an incubator containing 5% CO_2 at 37°C. After incubation with drugs, cells were fixed with 1% glutaraldehyde (Wako, Tokyo, Japan)/phosphate-buffered saline, and cell morphology was observed under a phase-contrast microscope.

2.4. MTT assay

1321N1 cells were seeded on 96-well plates (200 μl/well) at a density of 5×10^5 cells/ml. At the end of the experiment, MTT (0.1 mg) was added to each well and the plates were incubated for 4 h at 37°C. After centrifugation at $350 \times g$ for 5 min, the medium was replaced with dimethyl sulfoxide. The absorbance of reduced MTT at 595 nm was measured with a plate reader (Taglialatela et al., 1997).

2.5. Assay for neurite outgrowth

The neurite outgrowth of PC-12 cells observed under a phase-contrast microscope was regarded as a sign of differentiation. The differentiation of PC-12 cells was scored as follows: cells without neurite outgrowth were scored 0, cells bearing neurites as long as one cell diameter were scored 1, cells bearing neurites two to three times longer in length than their diameter were scored 2, and cells bearing neurites that were extremely long or which formed a synapse were scored 3. The mean differentiation score was obtained for 70 PC-12 cells in each well. Data are expressed as means ± S.E.M. of the values of three to four wells.

2.6. Enzyme immunoassay of NGF

To determine NGF, 1321N1 cells were seeded into 24-well multiplates and allowed to grow to confluence. The day before the incubation with drugs, the medium was replaced with serum-free DMEM. After the cells were washed, drugs in DMEM/1% bovine serum albumin without fetal calf serum were added to the well. The cells were cultivated for 24 h, and 500 μl of the condition medium was collected. The NGF content in the medium was measured by using a sandwich ELISA according to the instructions of NGF ELISA kit.

2.7. NGF gene expression

The total RNA from 1321N1 cells was extracted by using a total RNA extraction kit and semi-quantitative RT-PCR was carried out by using a RT-PCR kit. The sense primer (5′-CTT CAG CAT TCC CTT GAC AC-3′, 316–335 of human NGF cDNA) and the antisense primer (5′-AGC CTT CCT GCT GAG CAC ACA-3′, 889–909) were complementary to conserved regions of the cDNA from both mouse and human NGF (Amand et al., 1996). The NGF cDNA fragment was amplified 28 cycles (94°C for 60 s, 60°C for 30 s and 72°C for 50 s). The number of cycles that yielded a quantitative amount of product was determined in a preliminary experiment. Glyceraldehyde 3-phosphate dehydrogenase (G3PDH) transcripts were used as a positive control. PCR products, which were separated

by electrophoresis on 2% agarose gels and stained with ethidium bromide, were analyzed with an image scanner (Foto/Eclipse, Fotodyne, Hartland, WI, USA).

2.8. Statistical methods

Data are expressed as mean values with ±S.E.M., and the significant of differences was analyzed with unpaired Student's *t*-test.

3. Results

3.1. Effects of scabronines A and G on the secretion of neurotrophic factors from 1321N1 cells

To investigate the biological effects of scabronines A and G (Fig. 1), 1321N1 cells were incubated for 2 days in DMEM containing 5% fetal calf serum supplemented with 100 μM scabronine A or G, and then PC-12 cells were cultivated for 2 days in the conditioned 1321N1 culture medium. Neither scabronine (A or G) was cytotoxic at concentrations up to 1 mM, determined by MTT assay (data not shown). The culture medium conditioned by 100 μM scabronine A or G enhanced neurite outgrowth (Fig. 2). Neither diterpenoid directly caused neurite outgrowth in PC-12 cells (data not shown). Therefore, it is assumed that the diterpenoids caused 1321N1 cells to release neurotrophic factors.

3.2. Effects of scabronines A and G on NGF secretion and NGF mRNA expression

NGF in the culture medium was measured after 1321N1 cells were incubated with scabronine A or G in serum-free DMEM/1% bovine serum albumin for 24 h (Fig. 3). These compounds significantly augmented NGF release from 1321N1 cells in a concentration-dependent manner.

In addition, NGF gene expression in 1321N1 cells was examined by using semi-quantitative RT-PCR methods. 1321N1 cells were incubated with 100 μM scabronine A or G in serum-free DMEM/1% bovine serum albumin for 4 h. The total RNA from 1321N1 cells was reverse

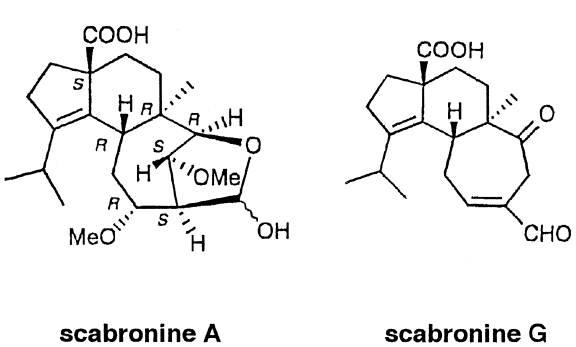

Fig. 1. Chemical structures of scabronine A and G.

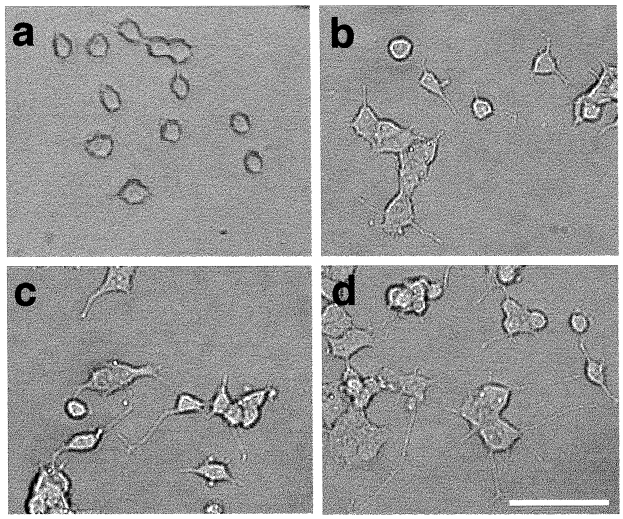

Fig. 2. Effects of scabronine A or G on glial cell-mediated morphological changes in PC-12 cells. 1321N1 cells (5×10^5 cells/ml) were incubated for 2 days in DMEM containing 5% fetal calf serum with 100 μM scabronine A or G, and then PC-12 cells were cultivated for 2 days in the conditioned 1321N1 cell culture medium. Phase-contrast microscopy of PC-12 cells after addition of DMEM (a), 1321N1 cell culture medium conditioned by 0.1% dimethyl sulfoxide (DMSO) as a vehicle of the scabronines (control) (b), scabronine A (c) or scabronine G (d). Scale bar: 100 μm.

transcribed, followed by 28 cycles of PCR using the specific primers to NGF cDNA (Fig. 4). When the density

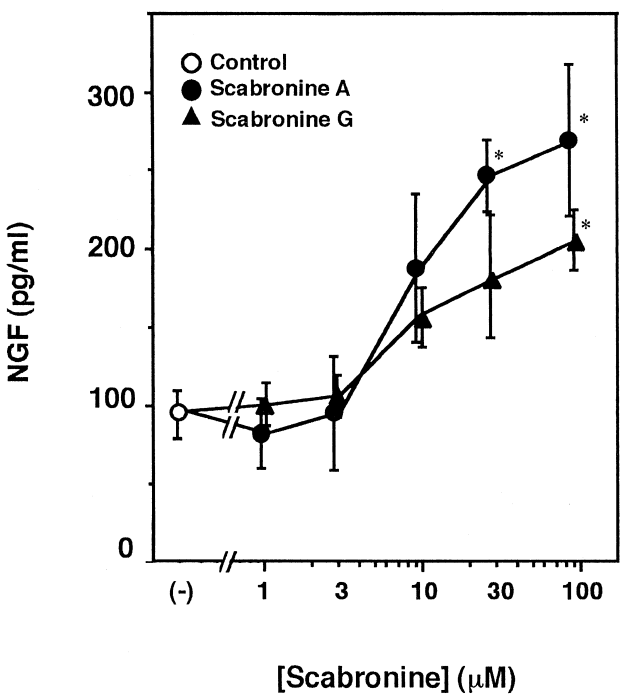

Fig. 3. Effects of scabronine A or G on the secretion of NGF from 1321N1 cells. After incubation with the indicated concentrations of the compounds for 24 h, NGF released from 1321N1 cells was measured using an ELISA. Control (○), scabronine A (●) and scabronine G (▲). Values are the means ± S.E.M. of three determinations. Scabronine A and scabronine G significantly increased NGF secretion compared with the corresponding control ($^*P < 0.05$).

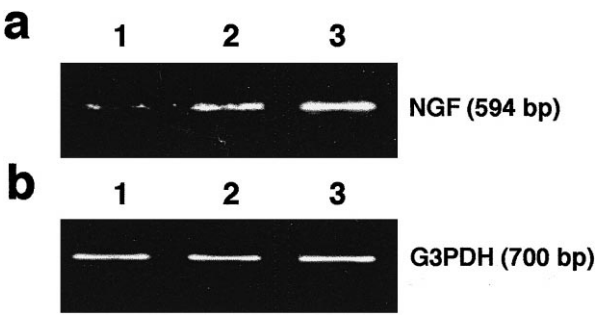

Fig. 4. Semi-quantitative RT-PCR analysis of NGF mRNA expression in 1321N1 cells. The cells were stimulated by the compounds for 4 h, and then total RNA from 1321N1 cells was reverse transcribed, followed by PCR as described in Section 2. (a) NGF (594 bp). (b) G3PDH (700 bp). Lanes 1, control; 2, 100 μM scabronine A; and 3, 100 μM scabronine G. Data are representative of three separate experiments.

of the band corresponding to the NGF cDNA fragment was normalized by that of G3PDH, NGF mRNA from these separate experiments was increased 3.17-fold by scabronine A and 3.58-fold by scabronine G. The results clearly demonstrated that NGF gene expression in 1321N1 cells was increased by the scabronines A and G.

3.3. Effect of NGF antibody on the neurite extension of PC-12 cells

To clarify whether the amounts of NGF secreted from 1321N1 cells in response to scabronine A or G were sufficient to cause the differentiation of PC-12 cells, we performed experiments with neutralizing antibody against NGF. After the culture media conditioned by scabronine A or G for 2 days was incubated with the neutralizing antibody (0.2 μg/ml) overnight at 4°C, PC-12 cells were cultivated in the media for 2 days. PC-12 cells still differentiated in the culture media, but less than in control cultures, whereas the differentiation induced by NGF (50 ng/ml) was completely abolished by the antibody (Fig. 5). Furthermore, NGF in the concentration range 125 to 1000 pg/ml, which corresponds to the amount of NGF secreted following stimulation with scabronine A or G, had no apparent effect on PC-12 cells (Fig. 6). These results suggest that the differentiation of PC-12 cells induced by scabronines A and G was due to the synergistic effect of plural neurotrophic factors including NGF.

3.4. Effect of PD98059 on the neurite extension of PC-12 cells

We have shown that 1321N1 cells secrete the new, low-molecular-weight neurotrophic factor which causes the differentiation of PC-12 cells through the activation of mitogen-activated protein kinase (MAPK) (Obara et al., 1998). We therefore carried out an experiment with the MAPK kinase (MEK) inhibitor PD98059. 1321N1 cells

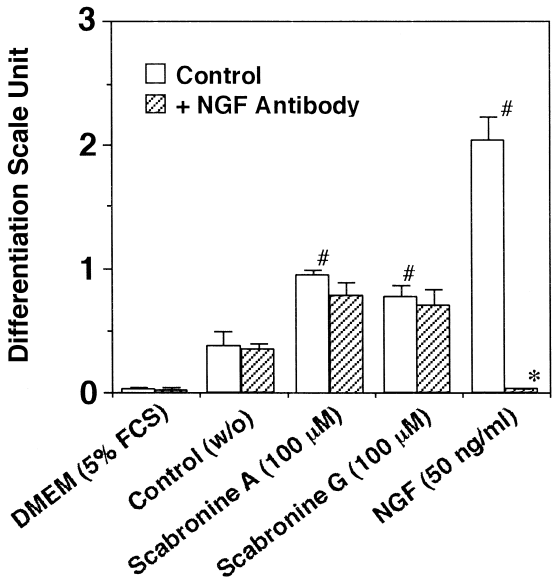

Fig. 5. Effects of the neutralizing antibody against NGF on the neurite extension of PC-12 cells. After 1321N1 cell culture media conditioned by scabronine A, scabronine G or NGF (50 ng/ml) was incubated with the antibody (0.2 μg/ml) overnight at 4°C, PC-12 cells were cultivated in the antibody-treated media for 2 days. The differentiation of PC-12 cells was evaluated as described in Section 2. Values represent the means ± S.E.M. for three to four wells. Scabronine A, scabronine G and NGF significantly accelerated differentiation as compared with control ($^{\#}P < 0.05$). The antibody treatment significantly reduced the differentiation compared with the stimulant alone ($^{*}P < 0.05$).

were incubated with scabronine A or G for 2 days, and then PC-12 cells were cultivated for 2 days in the condi-

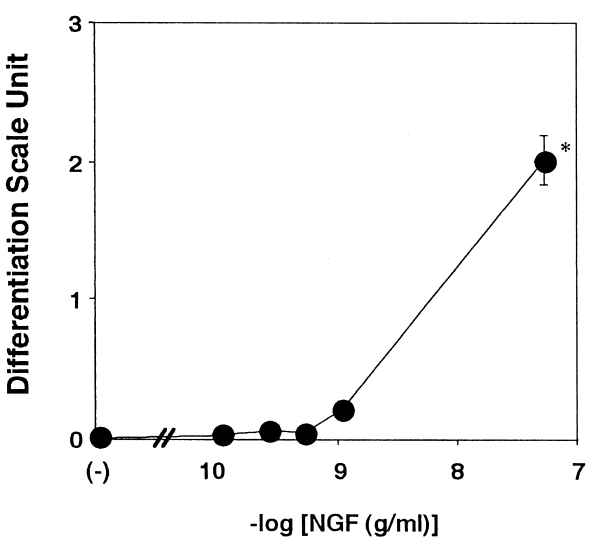

Fig. 6. Effect of NGF on neurite outgrowth from PC-12 cells. PC-12 cells were cultivated for 2 days in DMEM containing 5% fetal calf serum supplemented with NGF at the indicated concentrations. The differentiation of PC-12 cells by NGF was evaluated as described in Section 2. Values represent the means ± S.E.M. for three wells. An asterisk indicates a value significantly different ($^{*}P < 0.05$) from the corresponding value without NGF.

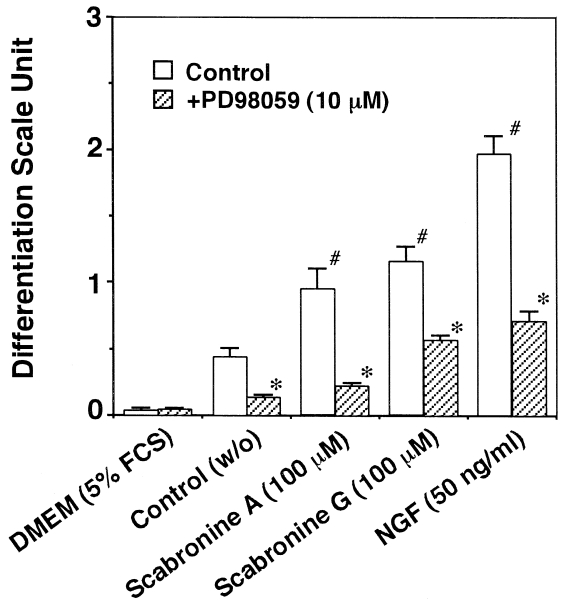

Fig. 7. Effect of PD98059 on neurite outgrowth from PC-12 cells. After 1321N1 cells were incubated with scabronine A or scabronine G (100 μM) for 2 days, PC-12 cells were cultivated in the conditioned media or in DMEM containing NGF (50 ng/ml) in the presence or absence of PD98059 (10 μM) for 2 days. The differentiation of PC-12 cells was evaluated as described in Section 2. Values represent the means ± S.E.M. for three to four wells. Scabronine A, scabronine G and NGF significantly accelerated differentiation as compared with the control ($^{\#}P < 0.05$). PD98059 significantly reduced differentiation as compared with the stimulant alone ($^{*}P < 0.05$).

tioned media containing PD98059 (Fig. 7). The enhancement of neurite extension by the conditioned media was significantly inhibited by PD98059.

4. Discussion

It has been suggested that exogenously administered NGF serves as a neurotrophic factor in the brain, preventing neuronal death and activating neuronal function (Hayashi, 1996). However, it is difficult and inconvenient to administer NGF by intracerebral infusion, and peripherally administered NGF does not cross the blood-brain barrier. This is why low-molecular-weight NGF inducers will be useful drugs for serious neuronal disorders such as Alzheimer's disease. Some low-molecular-weight NGF inducers have been already reported, such as catecholamines (Furukawa et al., 1986), active metabolite vitamin D (Musiol and Feldman, 1997; Veenstra et al., 1998), adenosine (Heese et al., 1997), benzoquinones (Takeuchi et al., 1990; Emmett et al., 1997), hericenones (Kawagishi et al., 1993), fellutamides (Yamaguchi et al., 1993b), kansuinin A (Yamaguchi et al., 1994), pyrroloquinoline quinones (Yamaguchi et al., 1993a) and erinacines (Kawagishi et al., 1996). Erinacines, which are isolated from *Hericium erinaceum*, are diterpenoids with a cyathane structure (Allbutt et al., 1971) similar to that of the scabronines used in the present study.

In the present study, we clearly demonstrated that the new diterpenoids, scabronine A and scabronine G, stimulated NGF synthesis in 1321N1 cells. However, the amount of NGF secreted into the culture media after incubation with scabronine A or G was not sufficient to promote the differentiation of PC-12 cells, which suggests that some other neurotrophic factors participate in the differentiation of PC-12 cells in addition to NGF, i.e., the differentiation of PC-12 cells induced by scabronine A and scabronine G was due to the synergistic effects of plural neurotrophic factors including NGF. In fact, expression of mRNA for interleukin-6 was also enhanced by scabronines in 1321N1 cells (Obara et al., unpublished observation). Interleukin-6 is a major neurotrophic factor that is released by astrocytes and which promotes the survival (Umegaki et al., 1996) and differentiation (Satoh et al., 1988) of PC-12 cells. In addition, we have recently shown that 1321N1 cells release a novel low-molecular-weight neurotrophic factor, but this factor has not been purified yet (Obara et al., 1998). The basal differentiation of PC-12 cells following incubation with conditioned medium from control cultures was assumed mainly to be attributed to this factor, which induces neurite extension in PC-12 cells by activating MAPK. Judging from the results shown in Fig. 7, the conditioned medium may contain the new neurotrophic factor. Further study is necessary to clarify the detailed mechanism of the action of the diterpenoids, scabronine A and scabronine G.

In conclusion, the scabronines A and G, isolated from *S. scabrosus*, potently stimulate the secretion of neurotrophic factors, including NGF. They are useful drugs to clarify the mechanisms underlying the synthesis and secretion of neurotrophic factors.

Acknowledgements

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