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Synthesis and Secretion of Nerve Growth Factor by Mouse Astroglial Cells in Culture

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Astroglial cells cultured from the mouse brain have been found to synthesize and secrete a material(s) with nerve growth factor-like immunoreactivity (NGF-LI) into their culture medium. A material(s) with NGF-LI showed identical properties to those of ßNGF purified from the mouse submaxillary gland in immunoreactivity, molecular weight, isoelectric point, and neurite outgrowth stimulatory activity. These results indicate that astroglial cells cultured from mouse brain are able to synthesize and secrete ßNGF in culture.

Nerve growth factor (NGF) is a protein that is required for the development and maintenance of sympathetic and some sensory neurons (1,2). In the peripheral nervous system, NGF is thought to be synthesized in the innervated end organs and retrogradely transported to the neuronal cell body (2,3). In fact, messenger RNA (mRNA) of NGF has been detected in the various end organs which are sympathetically innervated (4,5).

We have recently reported that fibroblast cells cultured from various end organs of the mouse synthesize and secrete a molecule identical to mouse submaxillary gland ßNGF (6,7). This observation suggests that the fibroblasts are to be included among the cells responsible for NGF synthesis in the peripheral organs. In the central nervous system (CNS), mRNA of NGF (4,8) and

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Abbreviations: EIA, enzyme immunoassay; GFAP, glial fibrillary acidic protein; NGF-LI, nerve growth factor-like immunoreactivity; CM, conditioned medium.

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low level of NGF (8) have been detected in the brain, suggesting that NGF acts as a neurotrophic factor in the CNS as in the peripheral nervous system (8). However, it is not yet established what kind(s) of cells in the brain are responsible for NGF synthesis and whether NGF in the brain is identical to that in the peripheral nervous system. Therefore, we have cultured cells from mouse brain and have attempted to clarify these problems.

MATERIALS AND METHODS

Materials: Mouse submaxillary gland ßNGF and anti-ßNGF antiserum were prepared as described previously (9). Dulbecco's modified Eagle's minimum essential medium (DMEM), fetal calf serum (FCS), and trypsin were obtained from Gibco; cell culture vessels, from Falcon; bovine serum albumin (BSA), from Armour; standard proteins for molecular weight determination, from Pharmacia; actinomycin D, from Makor; cycloheximide, from Nakarai; immunostaining kit for glial fibrillary acidic protein (GFAP), from Dako.

Cell culture: Whole brains of 8-day-old mice (ICR) were dissected out and cut into small pieces. The pieces were washed with Ca^{++} -, Mg^{++} -free phosphate-buffered saline (PBS), treated with 0.25 % trypsin at 37°C for 30 min, and triturated with a Pasteur pipet. The excess trypsin was removed by centrifugation at 200 x g for 5 min. The cells or cell clumps from one brain were cultured in a culture bottle (25 cm^2) with 2 ml of DMEM containing 10 % FCS, 50 μunits/ml of penicillin, and 50 μg/ml of streptomycin (DMEM-FCS) at 37°C in a humid atmosphere of 5 % CO_2 for 1 to 2 weeks with medium changes every 3 days. After confluence was reached, the cells in each bottle were dissociated by trypsin treatment and recultured in three new bottles. This procedure was repeated three times. The culture became composed of morphologically uniform cells. The cells were stained by the peroxidase anti-peroxidase antibody method using anti-human GFAP rabbit antiserum according to the manufacturer's instruction.

Two-site EIA: The experimental procedures and the specificity of the two-site EIA for measuring NGF have been fully described previously (9). The EIA system was not affected by DMEM-FCS and drugs used for experiments in this work.

Isoelectric focusing: Isoelectric focusing was carried out as described (6), except for the addition of Tween 20 (0.1 %) to the focusing solution in order to avoid adsorption of NGF onto the surface of the apparatus.

Bioassay: Neurite outgrowth stimulatory activity was tested using 8-day-old chick embryo dorsal root ganglia as previously described (6,9).

RESULTS

Cells were cultured from whole brains of 8-day-old mice. Primary cultures contained various types of cells with different morphologies. However, a few passages of the primary culture resulted in a fibroblast-like and morphologically uniform cell population (passaged culture). In order to identify cell types, the primary and the passaged culture were stained by the immunoperoxidase technique using antiserum to GFAP, a specific marker of

astroglia (10). The percentages of positively stained cells in the primary and the passaged cultures were 58.7 ± 7.0 % (n=5) and 97.0 ± 3.6 %, respectively. This indicated that the passaged culture was almost uniformly composed of astroglial cells while the primary culture contained cells of other types. Therefore, we hereinafter have referred to the cells of the passaged cultures as astroglial cells.

The astroglial cells were cultured for various times and the amounts of a material(s) with NGF-LI in the conditioned medium (CM) was measured by the two-site EIA. The CM was collected at the culture times of 0, 2, 4, 6, and 8 hr. Longer-term cultures of more than 8 hr were not analyzed because of the marked increase in cell number, which might complecate the results due to changes in the culture conditions. The experiment was started at a cell density of 2.0×10^5 cells/2.1 cm^2 well and ended in 8 hr at that of 3.1×10^5 cells/well. The concentration of a material(s) with NGF-LI in the CM linearly increased with the increase in culture time (Fig.1). The rate of NGF-LI secretion was about 5 pg/hr/10^5 cells. Next, the effects of actinomycin D, an inhibitor of RNA synthesis, or cycloheximide, an inhibitor of protein synthesis, on NGF-LI content in the CM were investigated. When the

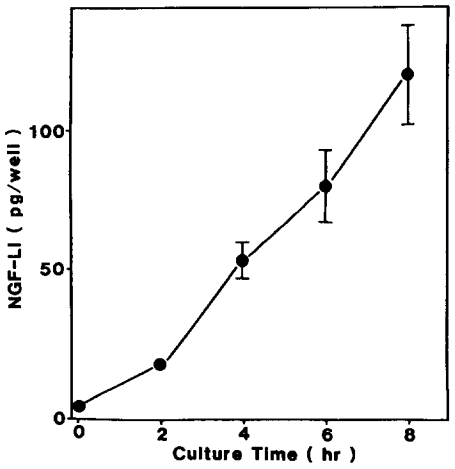

Fig.1 Time course of NGF-LI secretion by mouse astroglial cells.
When the cells reached a density of 2.0×10^5/well, the culture medium was changed to a fresh DMEM-FCS (0.5 ml). At various times thereafter the CM was collected and a 0.1-ml aliquot was used in the EIA for the measurement of NGF-LI. Each point is the mean ± S.E. of four determinations. The mean ± S.E. is not shown when it is less than the width of the symbol.

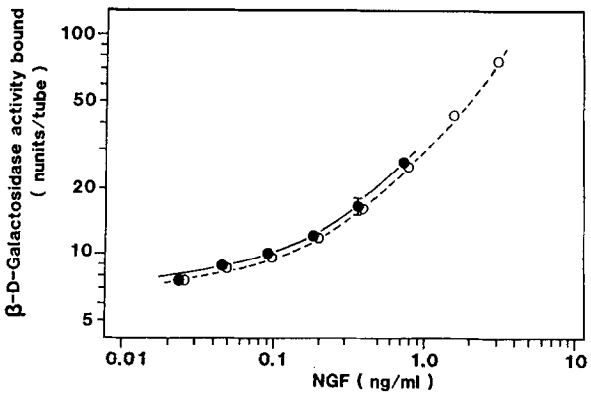

Fig.2 Antigenic relationship between the material(s) with NGF-LI in astroglial CM and mouse submaxillary gland βNGF.

Ten ml of astroglial CM (24 hr culture) or 0.5 ng of βNGF dissolved in 10 ml of DMEM-FCS was lyophilized. The dry residues were redissolved in 1 ml of the buffer (0.1 M phosphate buffer, pH 7.0, containing 0.3 M NaCl, 0.1 % BSA, 0.1 % NaN , and 1 mM MgCl). Both samples were serially diluted 3-fold with the buffer, and a 0.1-ml aliquot was used for the EIA. Immunoreactivity is expressed as ß-D-galactosidase activity bound to the tube. —●— astrocyte CM; --o-- βNGF. Each point is the mean ± S.E. of four determinations. The mean ± S.E. is not shown when it is less than the width of the symbol.

astroglial cells were cultured for 24 hr in the presence of actinomycin D (20 μg/ml), or cycloheximide (20 μg/ml), the content of NGF-LI in the CM was reduced to 4.6 ± 0.4 % (n=4) or 22.1 ± 0.1 % (n=4) of the value in the absence of the drug, respectively. These results indicated that astroglial cells cultured from mouse brain synthesize a molecule(s) with NGF-LI by DNA transcription-dependent protein synthesis and secrete it into medium as observed in fibroblast cells cultured from the mouse heart (6).

The molecular properties of the material(s) with NGF-LI were investigated. The NGF-LI in the CM of the atroglial cells was compared with the immunoreactivity of βNGF by the EIA. The dose-response curve obtained with NGF-LI was identical to that of βNGF (Fig.2). Then, the molecular weight of the material(s) with NGF-LI was estimated by the gel filtration method. An elution profile from a Bio-gel P-100 column is shown in Fig.3. NGF-LI was eluted out as a symmetric peak at the position identical to that of βNGF. The position of βNGF, which was determined by a separate run under the same conditions, is indicated as an open arrow in Fig.3. From calibration of the column using marker proteins, the molecular weight of the material(s) with NGF-LI was estimated as 26 K daltons. The fractions with NGF-LI obtained by

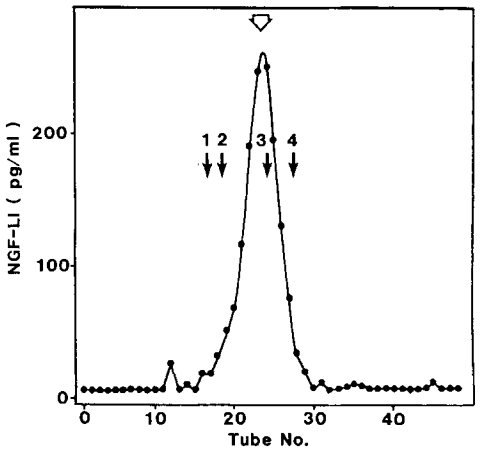

Fig.3 Gel filtration analysis of NGF-LI in astroglial CM on a column of Bio-gel P-100.

Fifty ml of astroglial CM (180 pg NGF-LI/ml; 24 hr culture) was lyophilized. The dry residue was redissolved in 5 ml of 0.1 M Tris-HCl buffer, pH 7.6, containing 1 M NaCl and applied to a Bio-gel P-100 column (2.5 x 103 cm) equilibrated with the same buffer. Fractions of 5 ml were collected at a flow rate of 30 ml/hr, and a 0.1-ml aliquot of each fraction was examined by EIA. Values are expressed as the mean of three determinations. Calibration of the column for estimation of the molecular weight of NGF-LI was performed using standard proteins: 1) BSA (molecular weight (Mr)=67 K); 2) ovalbumin (Mr=43 K); 3) chymotrypsinogen A (Mr=25 K); 4) RNAase A (Mr=13.7 K). The open arrow and the solid ones indicate the respective positions where purified βNGF and marker proteins were eluted.

Bio-gel P-100 column chromatography (tube nos.21-28) were pooled and concentrated with an Amicon PM-10 filter. The concentrate was used for

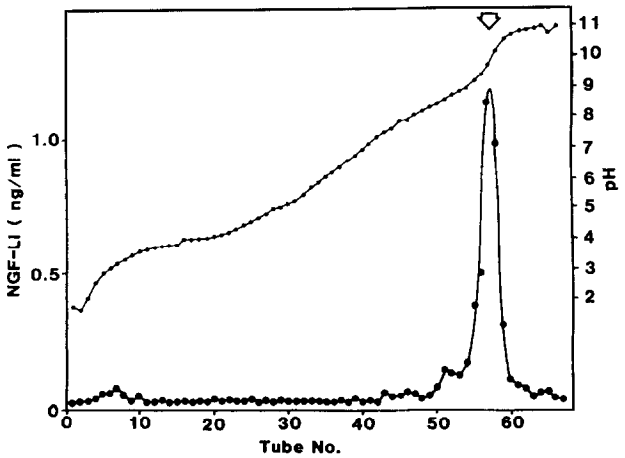

Fig.4 Isoelectric focusing analysis of NGF-LI.

Two ml of the concentrate (1.2 ng NGF-LI/ml) of the Bio-gel fractions with NGF-LI (tube Nos. 21-28 in Fig.3) was dialyzed against distilled water containing 0.1 % Tween 20 for 24 hr. The dialyzed sample was adjusted so as to contain 22.5 to 30 % sucrose and then applied at a point half the distance from the top of the column (2.1 x 20 cm). After electrophoresis at 800 V for 24 hr, fractions of 1 ml were collected, and a 25-μl aliquot of each was used for the EIA. Values are expressed as the mean of three determinations. The open arrow indicates the position where purified βNGF was focused.

isoelectric focusing analysis and the bioassay. Fig.4 shows the distribution of NGF-LI after isoelectric focusing analysis. NGF-LI became focused at the range between pH 9.2 and 10.5 similar to that of ßNGF (indicated as an open arrow in the figure). In the dorsal root ganglion bioassay, the concentrate of NGF-LI fractions (1.2 ng NGF-LI/ml; measured by the EIA) elicited a typical neurite outgrowth (+2 response). This level of activity was similar to that observed with purified ßNGF (9). These four criteria indicate that the material(s) with NGF-LI in the CM of mouse brain astroglial cells is a molecule identical to the ßNGF of the mouse submaxillary gland.

DISCUSSION

It has been reported that glial cells cultured from the CNS secrete various unidentified neurotrophic factors which act to support survival and growth of neurons (11-13). However, critical evidence for NGF secretion by CNS glial cells had not been presented until now. Only a clonal glioma cell line (rat C_6 glioma) has been reported to secrete NGF-like molecule(s), whose biological activity was diminished by the administration of anti-mouse submaxillary gland ßNGF antiserum (14). In this report, we have shown direct evidence that mouse brain astroglial cells synthesize and secrete a molecule identical to the ßNGF of the mouse submaxillary gland in immunoreactivity (Fig.2), molecular weight (Fig.3), isoelectric point (Fig.4), and neurite outgrowth stimulating activity. That is, NGF secreted by astroglial cells was found to be identical to NGF found in the CM of fibroblast cells cultured from the mouse heart (6). It is likely that NGF synthesis and secretion *in vivo* are attributed to fibroblast cells in the sympathetically innervated end organs and to astroglial cells in the brain, although the cellular composition and degree of differentiation of the cells in culture must be taken into consideration before reliable conclusions can be drawn.

The physiological significance of NGF synthesis by astroglial cells would appear to lie in its affording a proper supply of NGF to neurons for their growth, survival, and maintenance of function. NGF levels in the brain have been reported to be extremely low (8) (below 1.4 ng/g wet tissue weight),

although our present results show that the rate of NGF secretion by astroglial cells in culture is considerably high (about 5 pg/hr/10^5 cells). This discrepancy suggests that NGF synthesis is suppressed in the brain by some unknown mechanism(s).

In the present study, we used astroglial cells cultured from the whole mouse brain. It has been reported that the distribution of NGF and its mRNA is region-specific in the brain (8) and that astroglial cells cultured from different regions of the brain show different properties and functions (15,16). These observations present the possibility that the astroglial cells in different regions of the brain have different levels of ability for NGF synthesis. It is of great interest to study this possibility for understanding the functions of NGF in the brain.

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