J. Biochem. **120**, 865-868 (1996)

Identification of Interleukin-6 as a Factor That Induces Neurite Outgrowth by PC12 Cells Primed with NGF[1]

Sayoko Ihara,* Akihiro Iwamatsu,† Toshihide Fujiyoshi,* Akiko Komi,‡ Takao Yamori,‡ and Yasuhisa Fukui*,[2]

**Laboratory of Biological Chemistry, Department of Applied Biological Chemistry, Division of Agriculture and Life Science, The University of Tokyo, 1-1-1 Yayoi-cho, Bunkyo-ku, Tokyo 113;* †*Central Laboratories for Key Technology, Kirin Brewery Co., Ltd., 1-13-5 Fukuura, Kanazawa-ku, Yokohama, Kanagawa 236; and* ‡*Japanese Foundation for Cancer Research, 1-37-1 Kamiikebukuro, Toshima-ku, Tokyo 170*

Received for publication, September 2, 1996

We have screened for a factor that induces neurite outgrowth by PC12 cells only after NGF pretreatment in the supernatants of 38 transformed cell lines, finding three positive clones. The factor showing the strongest activity was purified and identified as IL6. In this NGF-IL6 system, the process of neurite outgrowth by PC12 cells can be separated into NGF-dependent and IL6-dependent steps. The IL6-dependent step requires RNA synthesis, suggesting that IL6 induces new gene expression depending on NGF-priming. These findings suggest that the gene expression during the differentiation process is regulated by at least two signals.

Key words: differentiation, IL6, neurite, NGF, PC12 cells.

Rat PC12 cells have been used as a good model system for neuronal differentiation. After incubation with nerve growth factor (NGF), they stop growing, form processes, and then exhibit other markers to show neuron-like characteristics such as electrical excitability after appropriate stimuli and the formation of synaptic-like vesicles (*1, 2*). The process of differentiation in which cells extend their neurites can be divided into several steps (*3*). After stimulation with NGF, second messengers are generated, and the signals may reach the nucleus and then the expression of immediate early genes will take place. Then the products of the immediate early genes may induce secondary or tertiary gene expression to accumulate the materials required for neurite outgrowth. At each step, continuous stimulation of NGF may be required, since removal of NGF results in apoptotic cell death instead of cell differentiation (*4*). It is likely that even after the secondary or tertiary gene expression started by the products of the immediate early genes, NGF controls the status of the cells in some way. Therefore, we hypothesized that the signal provided by NGF may be different at each step and that the signals required at certain steps could be provided by a factor other than NGF. In this study, we screened for factors which are capable of inducing differentiation only after cells have been primed with NGF for 2 h. The results suggest that the specific regulation of gene expression by extracellular factors occurs during the differentiation process.

PC12 cells were maintained in Dulbecco's modified minimal essential medium (DMEM) supplemented with 10% calf serum and 5% horse serum. They were treated with 20 ng/ml NGF in DMEM without serum for 2 h and then the medium was replaced with fresh medium containing 50% culture supernatants of various transformed cell lines. After 24 h, neurite outgrowth was examined. As shown in Table I, three of 38 samples gave positive results. Some others induced neurite outgrowth without NGF priming. The culture supernatant of one cell line, SF295 (*5*), exhibited strong activity only after NGF priming, so we purified and characterized the factor.

SF295 was cultured in DMEM without serum for 36 h and then the culture supernatant was collected, which was concentrated by ultrafiltration using a membrane with a cutoff of 5 kDa (Easy Flow; Sartorius) in a buffer comprising 2 mM Hepes-NaOH (pH 7.4) and 0.005% polyethylene glycol (PEG). PEG was added for all the procedures except the reverse phase chromatography to avoid non-specific binding to the columns. The sample was then mixed with CM Toyopearl (Tosoh, Tokyo) and incubated for 1 h at 4°C. The activity was eluted with a buffer comprising 5 mM Mes (pH 5.5) and 150 mM NaCl. The eluate was made to 50 mM NaCl and pH 5.8, and then mixed with Blue Toyopearl (Tosoh). After incubation for 1 h, the activity was eluted with a buffer comprising 10 mM Tris-HCl (pH 8.2) and 500 mM NaCl. The eluate was diluted to a NaCl concentration of 50 mM and then loaded onto a SynCropak CM300 column. The column was developed with a 60 ml linear gradient of NaCl (from 50 to 500 mM) in a buffer comprising 5 mM Mes-NaOH (pH 5.5). The activity was eluted at 140 to 160 mM salt. After the addition of 0.1% TFA, the sample was applied to a Resource RPC column (Pharmacia). Bound proteins were eluted with a continuous gradient of acetonitrile (from 0 to 90%) at the flow rate of 1 ml/min over 20 min. The activity was eluted at 60% acetonitrile

[1] This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas on "Functional Development of Neural Circuits," from the Ministry of Education, Science, Sports and Culture of Japan.
[2] To whom correspondence should be addressed. E-mail: ayfukui@hongo.ecc.u-tokyo.ac.jp
Abbreviations: IL6, interleukin-6; NGF, nerve growth factor; EGF, epidermal growth factor; TFA, trifluoroacetic acid; SDS, sodium dodecylsulfate; PAGE, polyacrylamide gel electrophoresis; PEG, polyethylene glycol; FGF, fibroblast growth factor.

(Fig. 1A). The overall recovery of the activity was about 2%. Laemmli's sample buffer (*6*) was added to each sample and acetonitrile was removed *in vacuo*. The samples were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 1B). The active fractions contained proteins with molecular sizes of 22 and 24 kDa. Finally, 0.7 μg of protein was obtained from 1 liter of the culture supernatant. The morphological change induced by the purified sample is shown in Fig. 2.

TABLE I. **Cell lines that secrete factors capable of inducing neurite outgrowth in PC12 cells.** A series of human tumor cell lines was screened for the ability to secrete factors capable of inducing neurite outgrowth. The tumor cell lines were cultured in serum-free medium for 24 h and then the culture supernatants were collected. PC12 cells were pretreated with or without NGF for 2 h and then exposed to fresh medium containing 50% culture supernatants. After 24 h, neurite formation was examined under a microscope.

	No. of cell lines tested	Neurites formed without NGF treatment	Neurites formed after NGF treatment
Lung	7	0	2
Colon	6	4	0
Stomach	6	0	0
Breast	6	0	0
Ovarian	5	0	0
Renal	2	0	0
Brain	6	1	1
Total	38	5	3

To determine which protein exhibits the activity, we tried to separate the two bands. Careful examination of the results of SynCropak CM300 column chromatography revealed that the upper band was eluted slightly earlier than the lower one (Fig. 1C). We therefore fractionated a small volume, and subjected the fractions containing one of the two bands to further purification on a Resource RPC column (Fig. 1, D and E). Both samples were equally active in the induction of neurite outgrowth suggesting that both the upper and lower bands contained activity.

These two bands were blotted onto a polyvinylidene difluoride (PVDF) membrane. Then the proteins were reduced and *S*-carboxymethylated as described before (*7*). After digestion with lysyl endopeptidase, the resulting fragments were separated by reverse phase chromatography and then subjected to amino acid sequencing for identification. The amino acid sequences, VAAPHRQP and QIRYILDGISALRK, were obtained for the upper band,

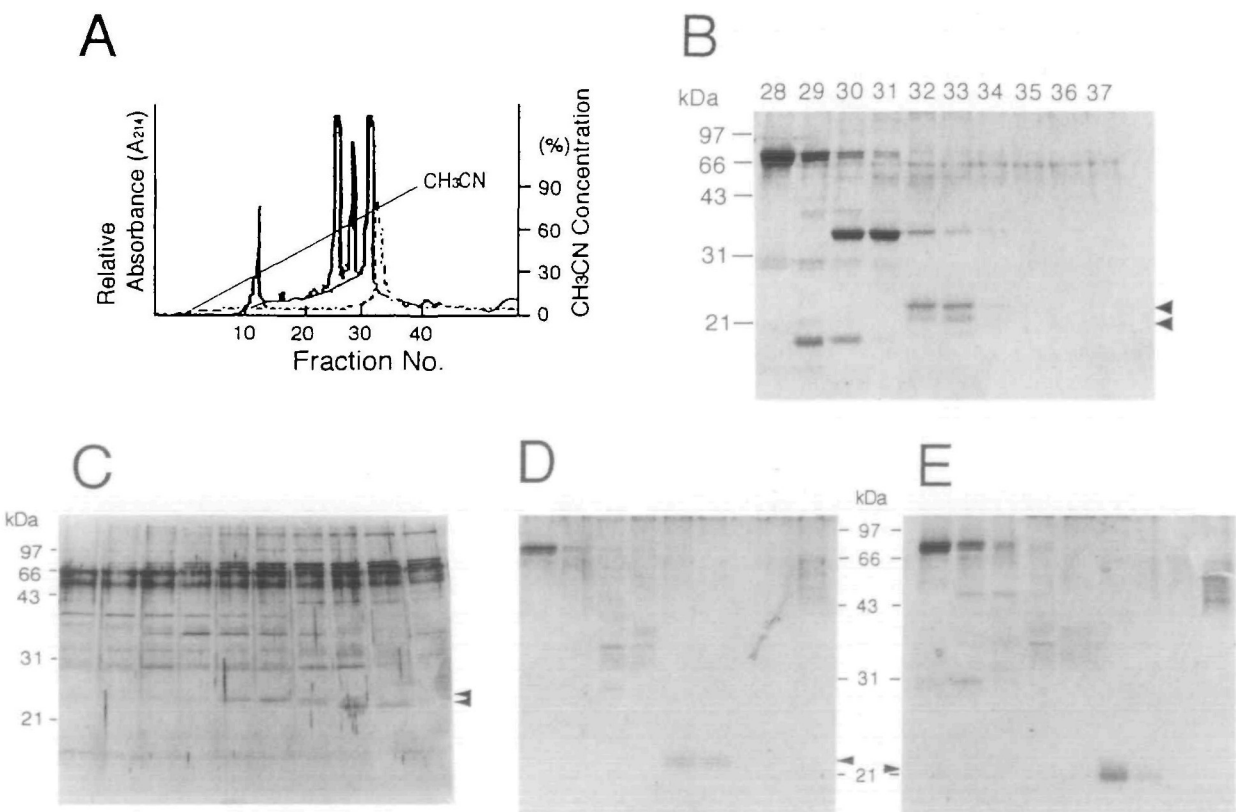

Fig. 1. **Purification of the differentiation factor on a Resource RPC column.** The active fractions obtained on SynCropak CM300 chromatography were applied to a Resource RPC column. This column was developed with a linear gradient of acetonitrile as described in the text. Panel A, elution profiles of protein (solid line) and activity (dashed line). Panel B, SDS-PAGE of the eluates from the Resource RPC column. Aliquots of the samples were mixed with Laemmli's sample buffer and then dried up to remove acetonitrile. They were reconstituted with an appropriate volume of water and then loaded onto a 14% acrylamide gel. The bands were visualized by Coomassie blue staining. Panel C, separation of the two bands by SynCropak CM300 column chromatography. The active fractions obtained with Blue Toyopearl were pooled and then loaded onto a SynCropak CM300 column. This column was developed with a linear gradient of NaCl, with a fraction size of 0.5 ml. SDS-PAGE of eluates from the SynCropak CM300 column is shown. Panel D, the fractions in panel C containing the only upper band were pooled and further purified on a Resource RPC column chromatography. Panel E, the lower band was purified and analyzed as in panel D. The arrowheads indicate the positions of bands which comigrated with the activity.

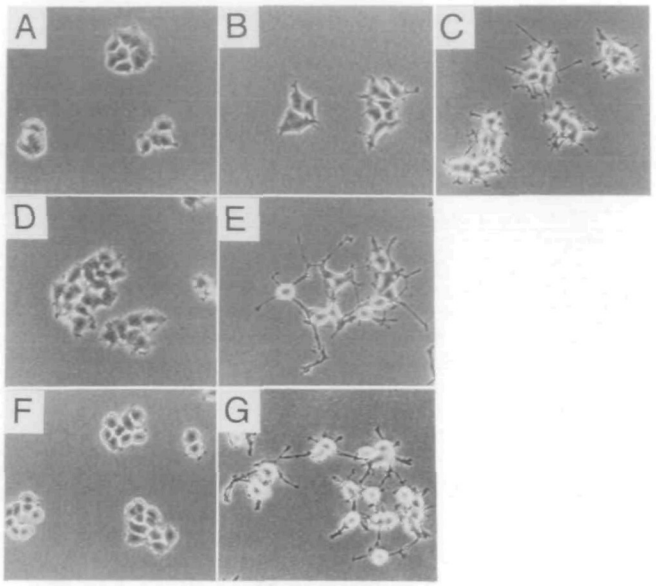

Fig. 2. **Neurite outgrowth on NGF-IL6 stimulation.** PC12 cells were pretreated with or without NGF (20 ng/ml) for 2 h. Then the medium was replaced with fresh medium containing 50% culture supernatant of SF295 cells or the purified IL6. A, control PC12 cells; B, pretreated with NGF and then changed to serum-free medium; C, treated with NGF throughout the incubation; D, culture supernatant of SF295 cells without pretreatment with NGF; E, supernatant of SF295 cells after pretreatment with NGF; F, purified factor without pretreatment with NGF; G, purified factor after pretreatment with NGF.

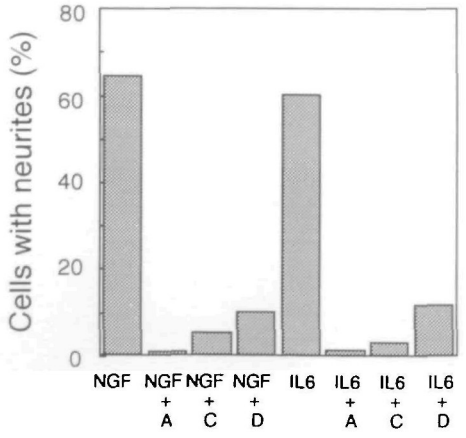

Fig. 3. **Inhibition of neurite outgrowth by RNA synthesis inhibitors.** PC12 cells were incubated with 20 ng/ml NGF. After 2 h, RNA synthesis inhibitors, 0.01 μM actinomycin D (A), 0.5 μM camptothecin (C), or 100 μM DRB (D), were added without a medium change (NGF) or with a change to IL6-containing medium (IL6). After 24 h, the cells with neurites longer than the cell bodies were counted.

and the sequences, DVAAPHRQP and NLDAITTPDP, for the lower one. They were identical to the sequences of human interleukin-6 (IL6) (*8*). Authentic IL6 exhibited the activity, confirming that the activity indeed resided in the two bands.

IL6 has been shown to induce neurite outgrowth by certain strains of PC12 cells without NGF (*9*). We have examined three strains of PC12 cells, but none of them

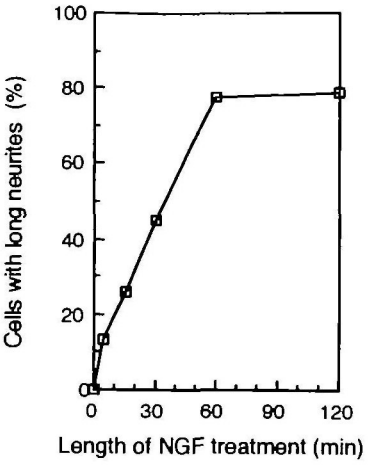

Fig. 4. **Length of NGF treatment required for neurite outgrowth due to IL6.** PC12 cells were incubated with NGF (20 ng/ml) for the periods indicated in the figure. Then NGF was removed and IL6 (20 ng/ml) was added. After 2 days, cells with neurites two times longer than their bodies were counted. Longer incubation with NGF results in more long neurites on the cells. Sixty minutes was enough for give the full effect.

responded to IL6 without NGF treatment. This was consistent with the recent report by Wu and Bradshaw (*10*).

It has been shown recently that IL6 can induce the regeneration of neurites by PC12 cells (*10*). The regeneration in PC12 cells is defined as neurite outgrowth after divesting of the neurites induced by treatment of the cells with NGF for more than 7 days (*10, 11*). Neurite outgrowth through regeneration is very fast and does not require RNA synthesis. It appears that the materials for regeneration are accumulated during the period of NGF stimulation before the divestiture. To distinguish the role of IL6 in this system from regeneration of neurites, the effects of actinomycin D, campthotecin, and 5,6-dichlorobenzimidazole riboside (DRB), which inhibit RNA synthesis through different mechanisms, were examined. After pretreatment with NGF for 2 h, the medium was replaced with fresh medium containing IL6 and one of the inhibitors. Neurite outgrowth was blocked suggesting that gene expression was required in the step (Fig. 3). Consistent with this result, NGF was not able to induce the differentiation of PC12 cells when RNA synthesis was inhibited at 2 h after the time of stimulation, suggesting that the cells did not accumulate enough material for neurite outgrowth within 2 h (Fig. 3). These results suggest that IL6 induces the gene expression required for neurite outgrowth. The gene expression may require NGF-priming since the cells did not respond to IL6 in the absence of NGF-priming. The priming could not be replaced with EGF or FGF, suggesting that NGF may generate some specific signal which is missing in the signal transduction of EGF or FGF.

The priming with NGF could be as short as 1 h. But shorter priming resulted in the formation of shorter neurites (Fig. 4). Increasing the dose of NGF to 100 ng/ml did not affect the results, ruling out the possibility of inefficient interaction of NGF with the cells. These results suggest that continuous stimulation with NGF for 1 h is required for establishment of the "primed" status. The effect of removal of NGF before IL6 stimulation was also

examined. IL6 had to be added within 30 min after removal of NGF (data not shown). The primed status was lost after further incubation in NGF-free medium, suggesting that continuous stimulation with NGF was required for maintenance of the primed status. It is not known what reaction induced by NGF is responsible for the establishment and maintenance of the primed status.

Overall, it appears that the process of differentiation of PC12 cells on NGF stimulation can be separated into at least two steps. One is NGF-specific and may be completed within 1 h. This step may not simply comprise the induction of certain genes since continuous stimulation of the cells with NGF was required for maintenance of the primed status. The other one can be replaced by IL6. This step may include production of the materials required for neurite formation. It has been suggested that NGF generates various signals such as those for activation of Ras, phospholipase C and phosphatidylinositol-3 kinase, which may have their own specific targets. However, analysis of the role of each signal in each step has not been performed because of the difficulty of separation of the steps in the process of differentiation. In this context, our system may be very useful for exploring the mechanism of differentiation of PC12 cells.

We thank Dr. Koutarou Kimura for critical reading of the manuscript and the helpful discussion.

REFERENCES

1. Greene, L.A. and Tischler, A.S. (1976) Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc. Natl. Acad. Sci. USA* **73**, 2424-2428
2. Dichter, M.A., Tischler, A.S., and Greene, L.A. (1977) Nerve growth factor-induced increases in electrical excitability and acetylcholine sensitivity of a rat pheochromocytoma cell line. *Nature* **268**, 501-504
3. Halegoua, S., Armstrong, R.C., and Kremer, N.E. (1991) Dissecting the mode of action of a neuronal growth factor. *Curr. Top. Microbiol. Immunol.* **165**, 119-170
4. Greene, L.A. (1978) Nerve growth factor prevents the death and stimulates the neuronal differentiation of clonal PC12 pheochromocytoma cells in serum-free medium. *J. Cell Biol.* **78**, 747-755
5. Rutka, J.T., Giblin, J.R., Hoifodt, H.K., Dougherty, D.V., Bell, C.W., MuCulloch, J.R., Davis, R.L., Wilson, C.B., and Rosenblum, M.L. (1986) Establishment and characterization of a cell line from a human gliosarcoma. *Cancer Res.* **46**, 5893-5902
6. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685
7. Iwamatsu, A. (1992) *S*-Carboxymethylation of proteins transferred onto polyvinylidene difluoride membranes followed by in situ protease digestion and amino acid microsequencing. *Electrophoresis* **13**, 142-147
8. Hirano, T., Yasukawa, K., Harada, H., Taga, T., Watanabe, Y., Matsuda, T., Kashiwamura, S., Nakajima, K., Koyama, K., Iwamatsu, A., Tsunasawa, S., Sakiyama, F., Matsui, H., Takahara, Y., Taniguchi, T., and Kishimoto, T. (1986) Complementary DNA for a novel human interleukin (BSF-2) that induces B lymphocytes to produce immunoglobulin. *Nature* **324**, 73-76
9. Satoh, T., Nakamura, S., Toga, T., Matsuda, T., Hirano, T., Kishimoto, T., and Kajiro, Y. (1988) Induction of neuronal differentiation in PC12 cells by B-cell stimulatory factor 2/interleukin 6. *Mol. Cell. Biol.* **8**, 3546-3549
10. Wu, Y.Y. and Bradshaw, R.A. (1996) Induction of neurite outgrowth by interleukin-6 is accompanied by activation of stat3 signaling pathway in a variant PC12 cell (E2) line. *J. Biol. Chem.* **271**, 13023-13032
11. Burstein, D.E. and Greene, L.A. (1978) Evidence for RNA synthesis-dependent and -independent pathways in stimulation of neurite outgrowth by nerve growth factor. *Proc. Natl. Acad. Sci. USA* **75**, 6059-6063