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REGULATION OF NEURITE OUTGROWTH THROUGH PROTEIN KINASE C AND PROTEASE NEXIN-1 IN NEUROBLASTOMA CELL

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INDEXING WORDS

neurite outgrowth; neuroblastoma; neurofilaments; serum depletion; thrombin; protein kinase C; protease nexin-1

SYNOPSIS

Accumulating evidence has demonstrated that protein kinase C (PKC) and protease nexin-1 (PN-1) may be involved in neuronal differentiation including migration, neurite outgrowth, target recognition, and synaptogenesis. We investigated the potential roles of PKC and PN-1 in neurite outgrowth of human neuroblastoma cell line, GOTO. Upon withdrawal of serum GOTO cells extended neurite processes within 3 h and formed fine network of neurites after 24 h. This morphological change was completely inhibited by thrombin and phorbol-12-myristate-13-acetate (PMA). Withdrawal of serum increased the neurofilament (NF)-L and -M mRNA levels and thrombin did not inhibit the effect of withdrawal of serum. A potent PKC inhibitor, H-7 induced neurite outgrowth in the presence of serum, however, it did not increase the NF mRNA levels. Actinomycin D and cycloheximide did not inhibit the initial neurite outgrowth induced by withdrawal of serum, while these inhibited the increase in the NF mRNA levels. Thrombin retracted the serum depletion-induced neurites but did not retract the neurites induced by H-7. The specific activity and subcellular localization of PKC did not differ between GOTO cells cultured in serum-containing and -free media for 12 h.

The serine protease inhibitory activity was undetectable in the serum-free conditioned medium of GOTO cells but the PN-1 mRNA was clearly detected by Northern blot analysis to a less extent than glial cells. Withdrawal of serum or treatment with H-7 did not increase the PN-1 mRNA level in GOTO cells, but thrombin increased its level about 7 folds in serum-free condition.

These results indicate that the initial neurite outgrowth requires neither new RNA nor protein synthesis, and that PKC negatively regulates neurite outgrowth and thrombin blocks neurite outgrowth through PKC-dependent pathways.

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INTRODUCTION

Some neuroblastoma cell lines undergo morphological and biochemical differentiation upon treatment with cAMP derivatives,[8, 22, 27] retinoic acid,[27, 28, 35] gangliosides,[31, 35] and serum depletion.[8, 25, 27, 35] Activator of PKC, PMA, inhibits neurite outgrowth induced by serum depletion.[13] Recently it has been reported that inhibitors of PKC, H-7 and staurosporine, induce extension of neurites and increase acethylcholine esterase activity in neuroblastoma cells.[7, 12, 18] Furthermore, gangliosides induce neurite outgrowth and down-regulation of the PKC mRNA.[33] These results strongly suggest that PKC negatively regulates neurite outgrowth of neuronal cells.

The balance between proteases and their inhibitors has been studied as another important regulator of neuronal differentiation. Thrombin, one of the strong serine proteases, reverts serum depletion-induced extension of neurites and the serine protease inhibitor, PN-1, promotes extension of neurites in neuroblastoma cells.[10, 11] PN-1 is secreted from a variety of cells including foreskin fibroblasts [24] and glial cells,[14, 20] and forms a tight complex with serine proteases such as thrombin, urokinase, and plasminogen activator, thereby inhibits the protease activity.[1, 15] Recently, it has been shown that some neuroblastoma cells express the PN-1 mRNA when examined by Northern blot analysis.[34] These results suggest that PN-1 may play a role in serum depletion-induced extension of neurites in neuroblastoma cells.

In this report we have examined the morphology and the changes in the NF and PN-1 mRNA levels in neuroblastoma cell line, GOTO cells, upon treatment with serum depletion, thrombin, or inhibitor of PKC to explore molecular mechanism of serum depletion-induced morphological differentiation of neuronal cells. And we found that withdrawal of serum induced the increase in the NF mRNA levels and that the morphological differentiation was not always accompanied by the increase in the NF mRNA levels. We have also shown that the initiation of the neurite outgrowth by withdrawal of serum or the PKC inhibitor requires neither RNA nor protein synthesis.

MATERIALS AND METHODS

Materials

Human neuroblastoma cell line, GOTO,[26] human glioblastoma cell line, A172, and C6 rat glioma cell line were obtained from Japanese Cancer Research Resources Bank (JCRB). H-7 (1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride) was purchased from Seikagaku Kogyo (Tokyo, Japan). Chromozym®TH (Tos-Gly-Pro-Arg-pNA) was from Boehringer Mannheim (Mannheim, Germany). Human thrombin, phorbol-12-myristate-13-acetate (PMA), actinomycin D (Act-D), and cycloheximide (CH) were from Sigma (St. Louis, MO). The Megaprime DNA Labelling System, [α-^{32}P]dCTP (110 TBq/mmole), and [γ-^{32}P]ATP (110 TBq/mmole) were from Amersham (Tokyo, Japan). Plasmids containing cDNA for human NF-L,

NF-M, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from American Type Culture Collection (Rockville, MD).

Cell Culture

GOTO, A172, and C6 cells were cultured in RPMI 1640 medium supplemented with 25 mM HEPES, 10% fetal bovine serum, and appropriate antibiotics at 37°C in humidified 5% CO_2-95% air atmosphere. Rat astrocyte primary culture was established from cerebral cortex of one-day old rat pups as described before.[16]

Determination of neurite outgrowth

GOTO cells were seeded at 5.0 x 10^4 cells in 60-mm collagen-coated dishes 48 h before experiments. The cells were washed in serum-free medium, and test media were added as indicated in the experiments. To test the effect of two agents, the first agent was added 3 h before the second one. The number of neurite-bearing cells was determined by scoring more than 600 cells from the three randomly chosen fields per dish. The definition of the neurite-bearing cells is displaying processes longer than one cell body length in diameter.

Northern blot analysis

Total RNA fraction was recovered from cultured cells by the acid-guanidine-phenol-chloroform (AGPC) method.[5] Northern blot analysis was performed as described previously.[32] In brief, the 1.5-kb EcoRI fragment of NF 5.1,[21] the 1.0-kb EcoRI and BamHI fragment of NF 11,[21] and the 1.2-kb EcoRI fragment of pHcGAP NR [30] were labeled with [α-^{32}P]dCTP by using the Megaprime DNA Labelling System to make a specific activity of 1-3 x 10^8 cpm/μg of DNA. After hybridization at 42°C for 18-24 h, the sheet was routinely washed in 2 x SSC at room temperature for 5 min twice and then in 2 x SSC with 0.1% SDS at 65°C for 30 min twice. The final wash for the PN-1 probe was done in 0.1 x SSC with 0.1% SDS at 65°C. The washed sheet was exposed to a Kodak X-OMAT AR film with an intensifying screen at -80°C.

Synthesis of cDNA of PN-1

Two oligonucleotides were designed, based on nucleotide sequence of human and rat PN-1,[17, 29] and synthesized on an automatic DNA synthesizer (Applied Biosystems Model 391). The nucleotide sequence of primers were as follows;
The forward primer: 5'-GCACAAACAGATTTGAAGGAG-3' (21bp)
(corresponds to nt. 898-918 in human sequence)
The reverse primer: 5'-ATGGATCCAATTGTAGGGACAACTTCGAAGG-3'
(31bp) (corresponds to 3'-non coding region nt. 267-290 in human sequence with attached HindIII restriction site)
These two nucleotides were then used as primers in reverse transcription-polymerase chain reaction

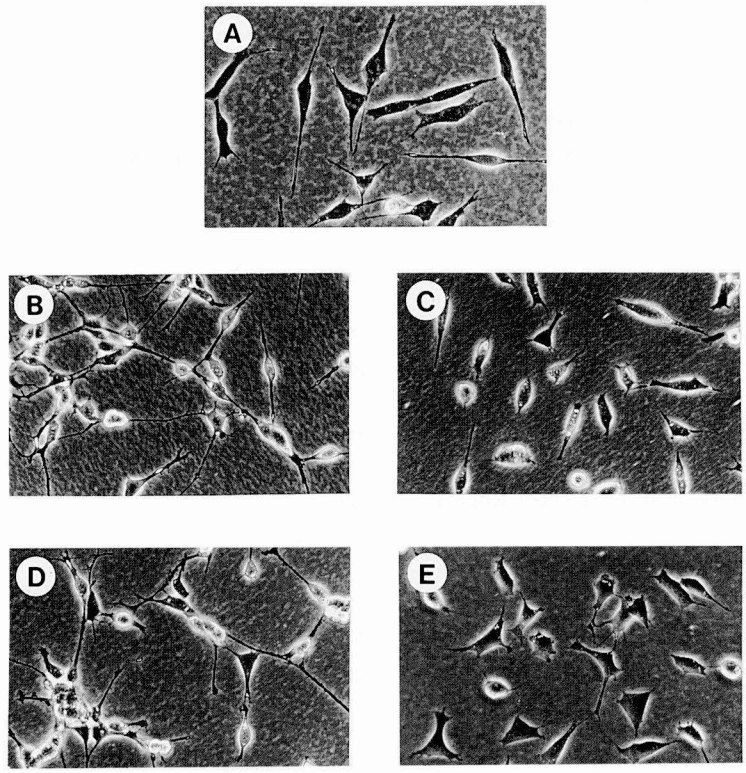

Fig. 1 Morphological changes in GOTO cells

Phase-contrast photomicrographs of GOTO cells cultured in various conditions for 24 h. A, serum-containing medium; B, serum-free medium; C, serum-free medium with thrombin (300 ng/ml); D, serum-containing medium with H-7 (25 μM); E, serum-free medium with PMA (10 ng/ml).

(RT-PCR). Agarose gel electrophoresis of the RT-PCR product gave a single 591-bp band, which was compatible to a calculated size. The RT-PCR product from GOTO cells with a 5'-side HindIII restriction site at nt. 1057 was ligated at HindIII site of a pGEM®-3Zf(+) plasmid (Promega, Madison, WI) and that of C6 cells was blunt-end ligated at SmaI site of the same plasmid and introduced into E. coli competent cells, JM-109 (Toyobo, Tokyo, Japan). The 429-bp HindIII fragment of human PN-1 cDNA, and 620-bp EcoRI and BamHI fragment of rat PN-1 cDNA from the plasmid were purified with GeneClean Kit (Bio 101, La Jolla, CA) and used as a species-specific cDNA probe in Northern blot analysis.

Determination of serine protease inhibitory activity

Conditioned media derived from 48-h serum-free cultures of GOTO, A172, and C6 cells, and rat astrocytes in primary culture (n=5) were concentrated using a CL-6B dextran-sulphate sepharose column which binds serpin family protein. Sepharose CL-6B (Sigma, St. Louis, MO) was coupled to dextran sulphate after epoxide activation of the resin as described by Farrell *et al.*.[6] The serine protease inhibitory activity was determined using thrombin and Chromozym®TH as described by Bartl *et al.*.[2] The activity was expressed as percentage compared with the sample from rat astrocyte in primary culture.

Determination of protein kinase C activity

GOTO cells cultured in serum-containing or serum-free medium for 12 h (n=3) were collected and sonicated for 10 sec three times in homogenizing buffer consisting of 50 mM Tris-HCl at pH7.5, 5 mM EDTA, 10 mM EGTA, 10 mM benzamidine hydrochloride (Nacalai Tesque, Kyoto, Japan), 0.3% 2-mercaptoethanol, and 50 μg/ml phenylmethylsulfonyl fluoride (PMSF). After the exclusion of nuclei and cell debris through 5,000 x g centrifugation, the extract was centrifuged for 60 min at 100,000 x g at 4°C and supernatant was recovered as the cytosolic fraction. The pellet was dissolved in homogenizing buffer with 1% Triton X-100 and chilled for 60 min on ice. Then the microsomal fraction was recovered as the supernatant through the same centrifugation condition. An aliquot from the cytosolic and microsomal fraction samples was assayed for PKC activity using PKC enzyme assay system (Amersham, UK) and [γ-^{32}P]ATP. Protein content in each sample was determined using BioRad protein assay system.

Others

Statistical analysis was performed by unpaired *t*-tests. Differences were considered significant if $p<0.05$.

RESULTS

Morphological changes of GOTO cells

Under serum containing condition, GOTO cells exhibit spindle-shaped appearance (Fig. 1A). Upon serum withdrawal GOTO cells extended long neurite processes as early as 3 h and the processes ended at the other processes or cell surface forming complex network by 24 h (Fig. 1B). The addition of thrombin at the concentration of 300 ng/ml (=0.3 NIH units/ml) completely inhibited these morphological changes induced by serum depletion (Fig. 1C), and thrombin also retracted the already extended processes induced by serum depletion (data not shown). Treatment with H-7 under serum-containing medium induced the extension of the processes in GOTO cells by 24 h (Fig. 1D). PMA (30 ng/ml) did not induce the extension of the processes, and completely inhibited neurite outgrowth induced by serum depletion in GOTO cells (Fig. 1E).

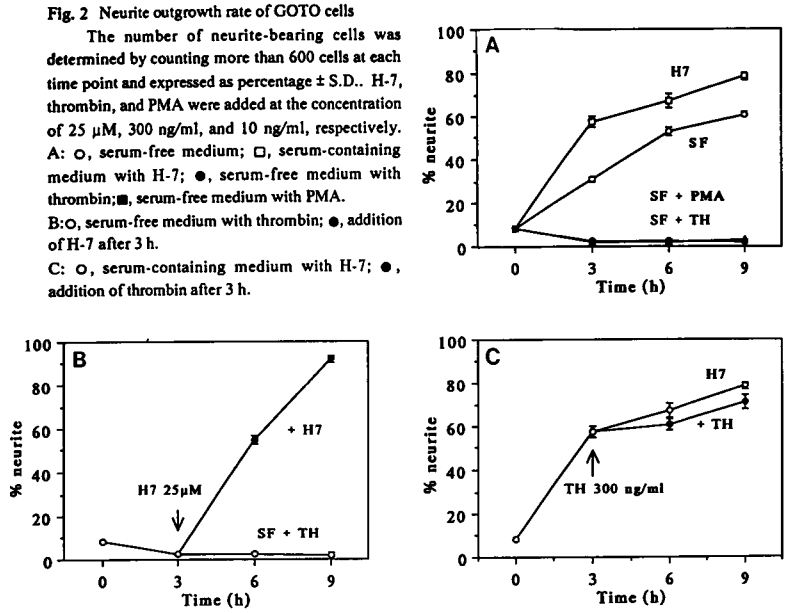

Fig. 2 Neurite outgrowth rate of GOTO cells

The number of neurite-bearing cells was determined by counting more than 600 cells at each time point and expressed as percentage ± S.D.. H-7, thrombin, and PMA were added at the concentration of 25 μM, 300 ng/ml, and 10 ng/ml, respectively. A: ○, serum-free medium; □, serum-containing medium with H-7; ●, serum-free medium with thrombin; ■, serum-free medium with PMA. B: ○, serum-free medium with thrombin; ●, addition of H-7 after 3 h. C: ○, serum-containing medium with H-7; ●, addition of thrombin after 3 h.

Neurite outgrowth

Serum depletion induced neurite outgrowth as early as 3 h and the number of neurite-bearing cells increased in a time-dependent manner within 6 h (Fig. 2A). After 9 h more than 60% of cells exhibited neurites under serum-depleted condition. H-7 induced neurite outgrowth more rapidly than serum depletion and approximately 80% of cells exhibited neurites after 9 h (Fig. 2A). Addition of thrombin or PMA completely inhibited the serum depletion-induced neurite outgrowth (Fig. 2A). We next examined the effect of Act-D and CH on the initial early neurite outgrowth induced by serum depletion and H-7 to test if this process requires new RNA or protein synthesis. One μg/ml of Act-D suppressed RNA synthesis to 1.8% of control and 10 μg/ml of CH suppressed protein synthesis to 18% of control in GOTO cells without affecting their viability. Addition of Act-D or CH to serum-depleted cultures did not affect the number of neurite-bearing cells after 3 h (Table 1). Under treatment with H-7 addition of Act-D and CH decreased the number of neurite-bearing cells approximately 10% ($p<0.05$) and 20% ($P<0.01$), respectively (Table 1).

We next examined the possibility that thrombin blocks neurite outgrowth through PKC dependent mechanism. When H-7 was added to the culture that had been treated for 3 h with thrombin under serum-free condition, it increased the number of neurite-bearing cells to 90% after 6 h (Fig. 2B). In the next experiment, we tested the effects of thrombin on the number of neurite-bearing cells that had been treated by H-7. When thrombin was added to the culture after 3-h treatment with H-7 under serum-containing condition, it did not affect the number of neurite-bearing cells induced by H-7 treatment (Fig. 2C).

Table 1. Effect of actinomycin D and cycloheximide on the initial neurite outgrowth in GOTO cells.

After 3 h from the start of treatment the number of neurite-bearing cells was determined by scoring more than 600 cells in each treatment. Values are expressed as mean ± S.D. of % neurite-bearing cells.

	Neurite outgrowth (% ± S.D.)
Serum-free	30.8 ± 0.4
+ Actinomycin D (1 μg/ml)	30.5 ± 2.1
+ Cycloheximide (10 μg/ml)	27.4 ± 2.6
H-7	57.5 ± 2.7
+ Actinomycin D (1 μg/ml)	47.0 ± 2.9 *
+ Cycloheximide (10 μg/ml)	38.5 ± 3.3 **

(n=3) * $p<0.05$; ** $p<0.01$

Changes in the NF-L and -M mRNA levels in GOTO cells

To examine whether those morphological changes are accompanied by an increase in the NF mRNA levels in GOTO cells, total RNA extracted from the cells cultured under various conditions were analyzed by Northern blot analysis. GOTO cells cultured in serum-containing medium expressed the 2.6- and 4.3-kb NF-L mRNA and the 3.5-kb NF-M mRNA to a small extent (Fig. 3). After 24-h treatment with serum depletion the NF-L and -M mRNA levels were increased by approximately 10 and 15 folds, respectively. In spite of complete inhibition of process extension, thrombin did not affect the increase in the NF mRNA levels induced by serum depletion. H-7 did not increase the NF mRNA levels after 24 h. Act-D and CH inhibited the increase in the NF mRNA levels induced by serum depletion to 95% and 60%, respectively. There was no significant change in the GAPDH mRNA level under these conditions (Fig. 3).

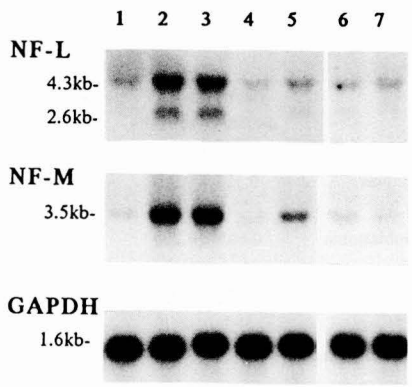

Fig. 3 Changes in the NF-L and -M mRNA levels in GOTO cells

Total RNA extracted from GOTO cells cultured in various conditions for 24 h was subjected to Northern blot analysis using NF-L, -M, and GAPDH probes. Lane 1, serum-containing control culture; lane 2, serum-free; lane 3, serum-free with thrombin (100 ng/ml); lane 4, serum-free with Act-D (1 μg/ml); lane 5, serum-free with CH (10 μg/ml); lane 6, same sample as lane 1; lane 7, serum-containing medium with H-7 (25 μM).

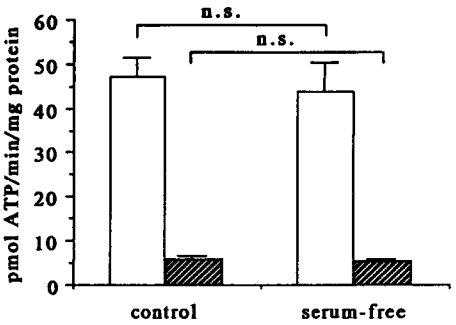

Fig. 4 PKC activity in GOTO cells cultured in serum-containing and -free media

PKC activity of the cytosolic fraction (open column) and microsomal fraction (hatched column) of GOTO cells cultured in serum-containing and -free media was determined as described under MATERIALS AND METHODS. The value is shown as mean ± S.D. from triplicate experiments.
n.s.; not significant.

The activity of PKC in GOTO cells

Since H-7 induced the neurite outgrowth in GOTO cells, we next examined the activity of PKC in GOTO cells cultured in serum-containing and -free media for 12 h to test if serum depletion-induced neurite outgrowth of GOTO cells is regulated by the change in the quantity of PKC. Approximately 80% of the PKC activity was recovered in the cytosolic fraction in GOTO cells, and there was no significant difference between the control and serum-free groups in the specific activity and subcellular localization (Fig. 4).

Serine protease inhibitory activity in serum-free conditioned media

Since there may be the involvement of PN-1 in the serum depletion-induced neurite outgrowth of neuroblastoma cells, we next examined if GOTO cells produce PN-1. Serine protease inhibitory activity in serum-free conditioned media derived from GOTO, A172, C6 cells, and rat astrocytes in primary culture was determined as described under MATERIALS AND METHODS. The serine protease inhibitory activity of GOTO cells was not detecetable. That of glial cell lines, A172 and C6, was much lower than that of rat astrocytes (Table 2).

Table 2. Serine protease inhibitory activity in serum-free conditioned media

Serine protease inhibitory activity in 48-h serum-free conditioned media from various cells was determined as described under MATERIALS AND METHODS. The mean activity of astrocytes is expressed as 100%.

	Serine protease inhibitory activity	
	$\Delta 405nm / 10^6$ cells	percent
astrocytes	0.193 ± 0.022	100%
A172 glioblastoma	0.044 ± 0.003	23%
C6 glioma	0.006 ± 0.002	3%
GOTO neuroblastoma	not detected	-

(n=5)

Expression of the PN-1 mRNA in various cells

We further examined the expression of the PN-1 mRNA in these cells by Northern blot analysis. When total RNA from GOTO, A172, and C6 cells was analyzed for the expression of the PN-1 gene, a 2.4-kb single band for PN-1 was clearly detected in these three cell lines. With the probe derived from rat gene (C6 cells), the PN-1 mRNA was detected in C6 glioma cells. With the probe derived from human gene (GOTO cells), the corresponding band for the PN-1 mRNA was detected more extent in A172 glioblastoma cells than in GOTO neuroblastoma cells (data not shown).

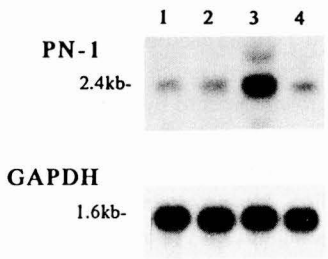

Fig. 5 Changes in the PN-1 mRNA level in GOTO cells

Total RNA (20 μg) extracted from GOTO cells was analyzed with the probes for human PN-1 and GAPDH. Lane 1, control serum-containing culture; lane 2, serum-free medium for 24 h; lane 3, serum-free medium with thrombin (100 ng/ml) for 24 h; lane 4, serum-containing medium with H-7 (25 μM) for 24 h.

Changes in the PN-1 gene expression in GOTO cells

We next examined the changes in the PN-1 mRNA level upon treatment with serum depletion, thrombin, and H-7. When GOTO cells were treated with serum depletion and H-7 for 24 h, no significant change in the PN-1 mRNA level was observed. On the other hand, addition of thrombin to serum-free culture increased the PN-1 mRNA level by approximately 7 folds (Fig. 5). There was no significant change in the GAPDH mRNA level under these experimental conditions.

DISCUSSION

Although number of agents are reported to induce morphological differentiation in neuronal cells, little is known about the mechanisms of regulating these changes. Serum depletion induces neurite outgrowth of neuroblastoma cells [8, 25, 27, 35] and cultured primary neurons,[3] however, underlying mechanism is also not known. We here reported that serum depletion increased the NF-L and -M mRNA levels in neuroblastoma cells, and thrombin retracted the extended neurites induced by serum depletion without affecting the NF mRNA levels. We have also shown that an inhibitor of PKC, H-7, also induced neurite outgrowth in GOTO cells without an increase in the NF mRNA levels. These results indicate that neurite outgrowth is not always accompanied by the increase in the NF mRNA levels. Indeed, Act-D and CH did not inhibit the initial neurite outgrowth within 3 h induced by serum depletion in GOTO cells (Table 1), while these compounds

suppressed the increase in the NF mRNA levels (Fig. 3). It was previously reported that neurite outgrowth is dependent on the assembly of microtubles and NFs from the pool of preformed protein subunits.[22, 25] But for prolonged neuritogenesis, it is indispensable to express the related genes to synthesize new cytoskeleton protein, such as microtubles and NFs.

It has been reported that inhibitors of PKC, staurosporine and H-7, induce neurite outgrowth in neuroblastoma cells [7, 12, 18] and activator of PKC, PMA, inhibits serum depletion-induced neurite outgrowth of neuroblastoma cells.[33] Furthermore, Baglioni *et al.*[12] recently reported that thrombin inhibits neurite outgrowth in rat neuroblastoma NB-2a cells through a PKC-dependent pathway. These results suggest that PKC negatively regulates neurite outgrowth in these cells. In GOTO cells serum depletion induces neurite outgrowth, and thrombin completely retracts the neurite processes induced by serum depletion (Figs. 1 and 2). And addition of H-7 decreases the ability of thrombin to block the neurite outgrowth induced by serum depletion (Fig. 2B). Furthermore, thrombin does not affect the neurite outgrowth induced by H-7 at all (Fig. 2C). These results suggest that thrombin inhibits neurite outgrowth through a PKC-dependent pathway in GOTO cells. However, since treatment with H-7 did not increase the NF mRNA levels, PKC does not seem to regulate these mRNA levels in this cell line. Moreover, since the specific activity and the localization of PKC in GOTO cells did not change significantly upon withdrawal of serum (Fig. 4), serum depletion-induced increase in the NF mRNA levels may not be linked to PKC pathways.

Several neuroblastoma cell lines and cultured sensory neurons extend neurite-like processes in serum-free condition. Several lines of evidence have suggested that the balance between proteases and protease inhibitors is critical on neurite outgrowth,[19] and PN-1 is involved in this process.[10, 11, 36] PN-1 was first identified in cultured human fibroblasts as a serine protease inhibitor [24] and later found to be identical with glia-derived neurite promoting factor.[9, 14] PN-1 is found almost exclusively in brain tissue,[23] especially in perivascular astrocytes and smooth muscle cells of arteries and arterioles,[4] and it has been proposed as a regulator of neurite outgrowth through anti-protease activities.[19] Addition of PN-1 to the culture medium promotes neurite extension in neuroblastoma cells [10, 11] and cultured sympathetic neurons,[36] and thrombin reverts this action.[10, 11] In GOTO cells we showed that serum depletion increased the NF mRNA levels (Fig. 3). And thrombin inhibited the serum depletion-induced neurite outgrowth without affecting the increase in the NF mRNA levels. These results show that the balance between proteases and protease inhibitors may be involved in the regulation of neurite outgrowth but this is not involved in the change in the NF mRNA levels.

Recently Cunningham *et al.*[34] have shown that a human neuroblastoma cell line, SK-N-SH, expresses the PN-1 mRNA to a lesser extent than human glioblastoma cells and fibroblasts when examined by Northern blot analysis. Serum-free conditioned medium from GOTO cells had no measurable serine protease inhibitory activity in our experimental condition (Table 2), but GOTO cells expressed the PN-1 mRNA to a detectable extent (Fig.5). Glial cells, such as C6 glioma and

A172 glioblastoma cells expressed the PN-1 gene transcripts much more than GOTO cells, and produced measurable amount of serine protease inhibitors in their conditioned media (Table 2). *In vivo* glial cells may produce the PN-1 and harmonize the balance between proteases and their inhibitors around neurons.

Thrombin increased the PN-1 mRNA level in GOTO cells cultured in serum-free medium (Fig. 5). Withdrawal of serum or H-7 did not affect its level. These results suggest that the increase of protease activity around neuronal cells or the mitogenic stimulation through thrombin may induce the increase in the PN-1 mRNA level within the neuronal cells themselves.

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3. The abbreviation used are: PKC, protein kinase C; PN-1, protease nexin-1; NF, neurofilament; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PMA, phorbol-12-myristate-13-acetate; Act-D, actinomycin D; CH, cycloheximide; RT, reverse transcription; PCR, polymerase chain reaction.

REFERENCES

1. Baker, J.B., Low, D.A., Simmer, R.L., and Cunningham, D.D.: Cell. 1980. 21. 37/45. Protease-nexin: a cellular component that links thrombin and plasminogen activator and mediates their binding to cells.
2. Bartl, K., and Lill, H.: Thromb. Res. 1980. 18. 267/272. A versatile assay method of antithrombin III using thrombin and Tos-Gly-Pro-Arg-pNA.
3. Bottenstein, J.E., Skaper, S.D., Varon, S.S., and Sato, G.H.: Exp. Cell Res. 1980. 125. 183/190. Selective survival of neurons from chick embryo sensory ganglionic dissociates utilizing serum-free supplemented medium.
4. Choi, B.H., Suzuki, M., Kim, T., Wagner, S.L., and Cunningham, D.D.: Am. J. Pathol. 1990. 137. 741/747. Protease nexin-1: localization in the human brain suggests a protective role against extravasated serine proteases.
5. Chomczynski, P., and Sacchi, N.: Anal. Biochem. 1987. 162. 156/159. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction.
6. Farrell, D.H., Van Nostrand, W.E., and Cunningham, D.D.: Biochem. J. 1986. 237. 907/912. A simple two-step purification of protease nexin.
7. Felipo, V., Minana, M.-D., and Grisolia, S.: J. Biol. Chem. 1990. 265. 9599/9601. A specific

inhibitor of protein kinase C induces differentiation of neuroblastoma cells.
8. Furmanski, P., Silverman, D.J., and Lubin, M.: Nature.1971. 233. 413/415. Expression of differentiated functions in mouse neuroblastoma mediated by dibutyryl-cyclic adenosine monophosphate.
9. Gloor, S., Odink, K., Guenther, J., Nick, H., and Monard, D.: Cell.1986. 47. 687/693. A glia-derived neurite promoting factor with protease inhibitory activity belongs to the protease nexins.
10. Gurwitz, D., and Cunningham, D.D.: Proc. Natl. Acad. Sci. USA 1988. 85. 3440/3444. Thrombin modulates and reverses neuroblastoma neurite outgrowth.
11. Gurwitz, D., and Cunningham, D.D.: J. Cell. Physiol. 1990. 142. 155/162. Neurite outgrowth activity of protease nexin-1 on neuroblastoma cells requires thrombin inhibition.
12. Herrick-Davis, K., Camussi, G., Bussolino, F., and Baglioni, C.: J. Biol. Chem. 1991. 266. 18620/18625. Modulation of neurite outgrowth in neuroblastoma cells by protein kinase C and platelet-activating factor.
13. Ishii, D.N., Fibach, E., Yamasaki, H., and Weinstein, I.B.: Science.1978. 200. 556/559. Tumor promoters inhibit morphological differentiation in cultured mouse neuroblastoma cells.
14. Knauer, D.J., Orlando, R.A., and Rosenblatt, D.: J. Cell. Physiol. 1987. 132. 318/324. The glioma cell-derived neurite promoting activity protein is functionally and immunologically related to human protease nexin-I.
15. Knauer, D.J., Thompson, J.A., and Cunningham, D.D.: J. Cell. Physiol. 1983. 117. 385/396. Protease nexins: cell-secreted proteins that mediate the binding, internalization, and degradation of regulatory serine proteases.
16. McCarthy, K.D., and De Vellis, J.: J. Cell Biol. 1980. 85. 890/902. Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue.
17. McGrogan, M., Kennedy, J., Li, M.P., Hsu, C., Scott, R.W., Simonsen, C.C., and Baker, J.B.: Bio/technol. 1988. 6. 172/177. Molecular cloning and expression of two forms of human protease nexin I.
18. Minana, M.-D., Felipo, V., and Grisolia, S.: Proc. Natl. Acad. Sci. USA 1990. 87. 4335/4339. Inhibition of protein kinase C induces differentiation in Neuro-2a cells.
19. Monard, D.: Trends Neurosci. 1988. 11. 541/544. Cell-derived proteases and protease inhibitors as regulators of neurite outgrowth.
20. Mulligan, L.P., Rosenblatt, D.E., Toms, R., and Johnson, D.: Neurosci. Lett. 1991. 128. 42/46. Protease nexin-1 activity in cultured Schwann cells.
21. Myers, M.W., Lazazarini, R.A., Lee, V.M.-Y., Schlaepfer, W., and Nelson, D.L.: EMBO J. 1987. 6. 1617/1626. The human mid-size neurofilament subunit.
22. Prasad, K.N., Sahu, S.K., and Sinha, P.K.: J. Natl. Cancer Inst. 1976. 57. 619/629. Cyclic nucleotides in the regulation of expression of differentiated functions in neuroblastoma cells.
23. Schurch-Rathgeb, Y., and Monard, D.: Nature.1978. 273. 308/309. Brain development influences the appearance of glial factor-like activity in rat brain primary cultures.
24. Scott, R.W., Bergman, B.L., Bajpai, A., Hersh, R.T., Rodriguez, H., Jones, B.N., Barreda, C., Watts, S., and Baker, J.B.: J. Biol. Chem. 1985. 260. 7029/7034. Protease nexin.

25. Seeds, N.W., Gilman, A.G., Amano, T., and Nirenberg, M.W.: Proc. Natl. Acad. Sci. USA 1970. 66. 160/167. Regulation of axon formation by clonal lines of a neural tumor.
26. Sekiguchi, M., Oota, T., Sakakibara, K., Inui, N., and Fujii, G.: Japan. J. Exp. Med. 1979. 49. 67/84. Establishment and characterization of a human neuroblastoma cell line in tissue culture.
27. Shea, T.B., Fischer, I., and Sapirstein, V.S.: Dev. Brain Res. 1985. 21. 307/314. Effect of retinoic acid on growth and morphological differentiation of mouse NB2a neuroblastoma cells in culture.
28. Sidell, N., Altman, A., Haussler, M.R., and Seeger, R.C.: Exp. Cell Res. 1983. 148. 21/30. Effects of retinoic acid on the growth and phenotypic expression of several human neuroblastoma cell lines.
29. Sommer, J., Gloor, S.M., Rovelli, G.F., Hofsteenge, J., Nick, H., Meier, R., and Monard, D.: Biochemistry,1987. 26. 6407/6410. cDNA sequence coding for a rat glia-derived nexin and its homology to members of the serpin superfamily.
30. Tso, J.Y., Sun, X.-H., Kao, T.-H., Reece, K.S., and Wu, R.: 1985. 13. 2485/2502. Isolation and characterization of rat and human glyceraldehyde-3-phosphate dehydrogenase cDNAs.
31. Tsuji, S., Arita, M., and Nagai, Y.: J. Biochem. 1983. 94. 303/306. GQ1b, a bioactive ganglioside that exhibits novel nerve growth factor-like activities in the two neuroblastoma cell lines.
32. Tsuneishi, S., Takada, S., Motoike, T., Ohashi, T., Sano, K., and Nakamura, H.: Develop. Brain Res. 1991. 61. 117/123. Effects of dexamethasone on the expression of myelin basic protein, proteolipid protein, and glial fibrillary acidic protein genes in developing rat brain.
33. Wada, H., Ohno, S., Kubo, K., Taya, C., Tsuji, S., Yonehara, S., and Suzuki, K.: Biochem. Biophys. Res. Commun. 1989. 165. 533/538. Cell type-specific expression of the genes for the protein kinase C family.
34. Wagner, S.L., Lau, A.L., Nguyen, A., Mimuro, J., Loskutoff, D.J., Isackson, P.J., and Cunningham, D.D.: J. Neurochem. 1991. 56. 234/242. Inhibitors of urokinase and thrombin in cultured neural cells.
35. Wu, G., Lu, Z.-H., and Ledeen, R.W.: Dev. Brain Res. 1991. 61. 217/228. Correlation of gangliotetraose gangliosides with neurite forming potential of neuroblastoma cells.
36. Zurn, A.D., Nick, H., and Monard, D.: Dev. Neurosci. 1988. 10. 17/24. A glia-derived nexin promotes neurite outgrowth in cultured chick sympathetic neurons.