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Glial fibrillary acidic protein is one of the key factors underlying neuron-like elongation in PC12 cells

MAKI SUGAYA-FUKASAWA*, TORU WATANABE*, MICHIKO TAMURA, SATSUKI EGASHIRA and HISASHI HISATOMI

Laboratory of Cellular and Molecular Biochemistry, Department of Materials and Life Science, Seikei Universty, Musashino-shi, Tokyo 180-8633, Japan

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Abstract. Nerve growth factor (NGF) normally induces the differentiation of PC12 cells into a neuron-like phenotype. In this study, we found that exposure of PC12 cells to a temperature of 42˚C for 24 h significantly decreased NGF-induced neurite elongation. Glial fibrillary acidic protein (GFAP) levels were decreased when PC12 cells were exposed to the heat stress, while PC12 cells overexpressing the gene encoding GFAP showed resistance. Mock-transfected PC12 cells exposed to NGF could be cultured for 72 h at 37˚C, whereas GFAP-transfected PC12 cells exposed to NGF could be cultured for over 100 h. Our results suggest that GFAP is necessary for the long-term maintenance of cells with a neuron-like phenotype.

Introduction

Glial fibrillary acidic protein (GFAP) is expressed in astrocytes in the central nervous system (CNS) (1) and is involved in many cellular functioning processes, such as cell structure and movement, cell communication and the functioning of the blood-brain barrier (2,3). It was found that the levels of several proteins expressed in astrocytes are altered in an Alzheimer's disease model (4,5), and cleavage of GFAP may contribute to astrocyte injury and damage in the Alzheimer's disease brain (6-8). GFAP plays an important role in mitosis, directly or indirectly, by adjusting the filament network present in the cell. During mitosis, the amount of phosphorylated GFAP increases and moves to the cleavage furrow (9). *In vitro*, astrocytes treated with antisense RNA against GFAP do not form a neuron-like phenotype (10). There are multiple disorders associated with inactive GFAP regulation, and mutations in the coding region of the GFAP gene are associated with Alzheimer's disease (11). Therefore, GFAP is thought to play crucial roles in the development and differentiation of astrocytes and is believed to be involved in the long-term maintainance of normal CNS myelination (12). However, the mechanisms by which GFAP is involved in the development and differentiation of astrocytes are not yet completely understood. To better understand the effect of GFAP expression on neurons, we generated stable PC12 cell lines overexpressing this gene.

The PC12 cell line, which was derived from a pheochromocytoma of the rat adrenal medulla (13), is the most common cell line used as neuron-like cells with neuron-like elongation abilities. Maintenance of the neuronal phenotype and survival of differentiated PC12 cells under serum-free conditions require constant nerve growth factor (NGF) exposure. PC12 cells stop dividing and terminally differentiate when treated with NGF. In the present study, PC12 cells with neuron-like elongation underwent apoptosis within 70-80 h. However, the PC12 cells overexpressing pGFAP from the plasmid underwent cell death 120 h after NGF exposure. The overexpression of GFAP delayed cell death in the PC12 cells.

Materials and methods

Cell culture. The PC12 cell line was purchased from the Japan Health Science Foundation (HSRRB, Osaka, Japan) and maintained in DMEM supplemented with heat-inactivated fetal bovine serum and horse serum. After culture for 48 h, the cells were treated with 1.0 μM NGF (Kyowa, Tokyo, Japan) for 0, 24, 48, 72, 96 or 120 h. All cells were grown in 5% CO_2 at 37˚C. The temperature was adjusted to 42˚C, and cells were observed. Apoptosis was detected by Annexin V and propidium iodide (PI) staining using flow cytometry (Quanta SC, Beckman Coulter, Fullerton, CA, USA).

RT real-time PCR analysis. Total RNA was extracted from the PC12 cells using the QuickGene RNA Cultured Cell HC Kit S (Fujifilm, Kanagawa, Japan) following the manufacturer's instructions. The final RNA preparations were resuspended in diethylpyrocarbonate-treated water and quan-

Correspondence to: Professor Hisashi Hisatomi, Laboratory of Cellular and Molecular Biochemistry, Department of Materials and Life Science, Seikei Universty, 3-3-1 Kichijoji, Kita-machi, Musashino-shi, Tokyo 180-8633, Japan
E-mail: hisatomi@st.seikei.ac.jp

*Contributed equally

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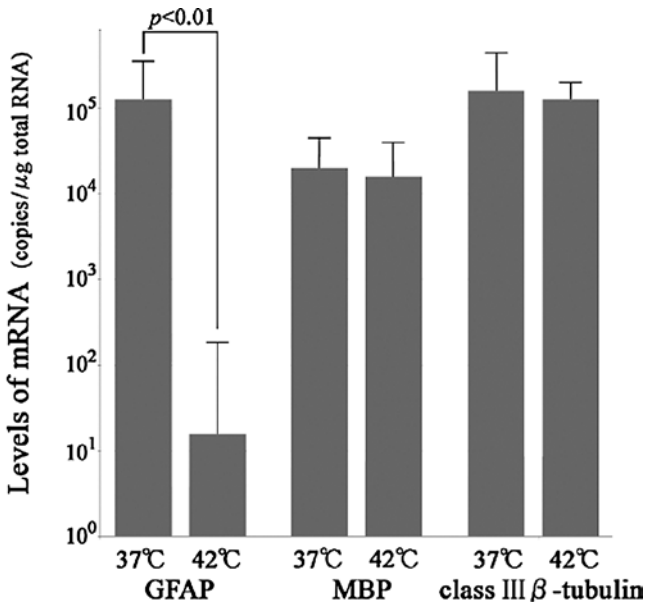

Figure 1. *GFAP*, *MBP* and *class III β-tubulin* mRNA in the PC12 cells exposed to NGF at 37 or 42˚C for 24 h. Triplicate RT real-time PCR analyses were performed as described in Materials and methods. As normalization to the *GAPDH* housekeeping gene is unreliable, mRNA expression levels are presented as the mRNA copy number per μg total RNA.

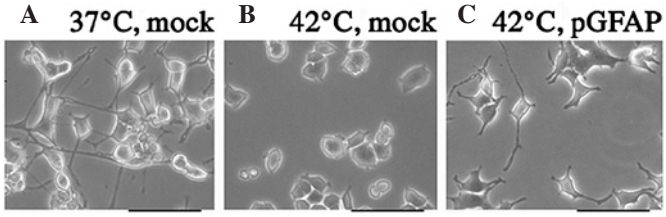

Figure 2. (A) Mock-transfected PC12 cells with NGF after 24 h in culture at 37˚C. (B) Mock-transfected cells with NGF after 24 h in culture at 42˚C. (C) pGFAP-transfected cells with NGF after 24 h in culture at 42˚C. The scale bar in each case represents 50 μm.

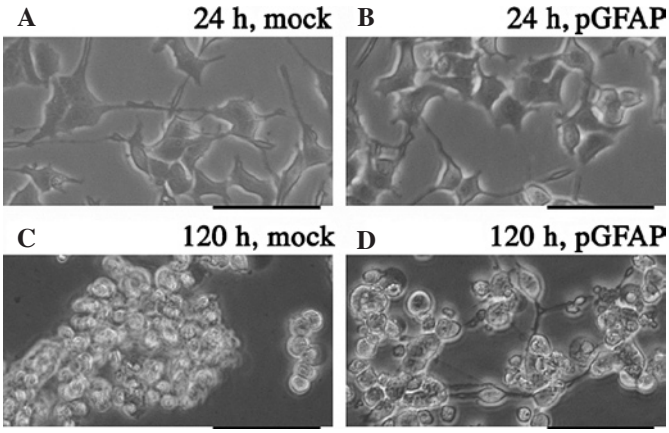

Figure 3. Determination of the transfection efficiency of pGFAP. (A) Mock-transfected PC12 cells with NGF in culture for 24 h at 37˚C. (B) pGFAP-transfected cells with NGF in culture for 24 h at 37˚C. (C) Mock-transfected cells with NGF in culture for 120 h at 37˚C. (D) pGFAP-transfected cells with NGF in culture for 120 h at 37˚C. The scale bar in each case represents 50 μm. Representative data from three independent experiments are shown.

tified by absorbance analysis at 260 nm. cDNA was prepared by incubating DNase-treated total RNA (0.1 μg) with M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA) in the presence of random primers (Invitrogen). The real-time PCR reaction mixture was prepared using a FastStart SYBR Green Master (Roche Diagnostics, Mannheim, Germany). The primer set for amplification of a *GFAP* mRNA was designed according to GenBank NM_017009, using a forward primer at the exon 2 region, 5'-CAA GAT GAA ACC AAC CTG AGG CT-3', and a reverse primer at the exon 4 region, 5'-GGC TTG GCC ACA TCC ATC T-3' (product length 221 bp). The primer set for amplification of a *myelin basic protein* (*MBP*) mRNA was designed according to GenBank M25889, using a forward primer at the exon 4-6 region, 5'-GGC AAG GAC TCA CAC ACR AGA ACT-3', and a reverse primer at the exon 7-8 region, 5'-GGG ACA GGC CTC TCC CCT T-3' (product length 154 bp). The primer set for amplification of a *class III β-tubulin* mRNA was designed according to GenBank AF459021, using a forward primer at the exon 2 region, 5'-ATA GAC CCC AGC GGC AAC TAT GTG-3', and a reverse primer at the exon 3 region, 5'-AGG CCT GAA TAG GTG TCC AAA GGC-3' (product length 174 bp). The real-time PCR reaction was carried out for 45 cycles (95˚C for 20 sec, 60˚C for 30 sec and 72˚C for 20 sec) using an iCycler iQ™ Real-Time Detection System (Bio-Rad, Hercules, CA, USA). *GAPDH* cDNA was amplified for normalization (Applied Biosystems, Foster City, CA, USA); mRNA levels are presented as the mRNA copy number per μg total RNA.

Overexpression of pGFAP. The gene encoding *GFAP* was subcloned into the mammalian expression vector pTriEX™-3 (Merck, San Diego, CA, USA), and the entire sequence was verified by DNA sequencing. For overexpression in PC12 cells, *GFAP* was subcloned from pTriEX™-3 vectors into NovaBlue Singles™ competent cells (Merck). The resultant plasmids were designated as pGFAP. Cells were seeded at a density of 10^6 cells per 10-cm dish and transfected with 8 μg of plasmid DNA using 120 μl of GeneJuice® Transfection reagent (Merck) for 2 h at 37˚C, after which time the DMEM medium plus 10% serum was changed. After 24 h, cells were cultured under serum-free conditions and exposed to NGF stimulation. PC12 cells that were stably transfected with cDNAs encoding GFAP or the empty vector (mock) were grown to confluence in DMEM.

Results

RT real-time PCR. The levels of *GFAP* mRNA in the culture at 37˚C ranged from $10^{4.6}$ to $10^{5.5}$ copies/μg total RNA and were evaluated as the mean ± SE ($10^{5.1}\pm10^{0.4}$) copies/μg total RNA. The levels of *GFAP* mRNA in the culture at 42˚C ranged from 0.0 to $10^{2.1}$ copies/μg total RNA and were evaluated as the mean ± SE ($10^{1.2}\pm10^{0.9}$) copies/μg total RNA; this difference was significant ($p<0.01$; Student's t-test). The levels of *MBP* mRNA in the culture at 37 and 42˚C were $10^{3.9}$-$10^{4.6}$ and $10^{3.8}$-$10^{4.6}$ copies/μg total RNA, respectively. The levels of *class III β-tubulin* mRNA in the culture at 37 and 42˚C were $10^{4.7}$-$10^{5.6}$ and $10^{4.9}$-$10^{5.3}$ copies/μg total RNA, respectively (Fig. 1).

Cell culture and overexpression of pGFAP. In the mock-transfected PC12 cells exposed to NGF, no apoptosis was induced at 0, 24 or 48 h at 37˚C. After 72 h, apoptosis was induced when the cell that expands the neurite was cultured at 37˚C (data not shown). Exposure of PC12 cells to a temperature of 42˚C for 24 h significantly decreased NGF-induced neuron-like elongation compared to cells cultured at 37˚C, while it was possible to maintain the neuron-like elongation at 42˚C in the cells with pGFAP overexpression (Fig. 2). Mock-transfected PC12 cells showed similar features to control PC12 cells when incubated at 42˚C. In cells overexpressing pGFAP, neuron-like elongation was maintained in the culture for 120 h (Fig. 3).

Discussion

In the present study, GFAP was found to play an important role, directly or indirectly, in the protection of *in vitro* PC12 cultured cells from damage. Unfortunately, apoptosis progresses rapidly and the relation between the level of GFAP and the degree of inhibition of apoptosis is not yet understood. However, it appears that the existence of GFAP inhibited apoptosis in the PC12 cells with neuron-like elongation. Heat stress at a temperature of 42˚C caused the PC12 cells to die. Neuron-like elongation was lost in the cultures at 42˚C, and these cells underwent apoptosis. Severe stress was found to play a role in the pathogenesis of neurodegenerative diseases, such as Alzheimer's disease. If neuronal cells can be made sufficiently resistant to stress, then the pathogenesis of neurodegenerative diseases should be slowed or decreased. Our results corroborate those of other researchers who have shown that GFAP is necessary for the long-term maintenance of the normal CNS (14-16).

The mechanism by which GFAP overexpression inhibits the apoptosis of PC12 cells is uncertain. However, we propose that this novel function of GFAP plays an important role in the ability of astrocytes to protect neuronal cells against apoptosis. These data support the concept that GFAP is responsible for many of the progressive astroglial changes that appear after CNS injury and disease. Further evaluation of this and other possible roles of GFAP, as well as the regulation of GFAP expression, are crucially important for the development of new strategies for maintaining homeostasis in the nervous system.

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