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Glial cell line-derived neurotrophic factor promotes proliferation of neuroglioma cells by up-regulation of cyclins PCNA and Ki-67

D.-W. QU, Y. LIU, L. WANG, Y. XIONG, C.-L. ZHANG, D.-S. GAO

Department of Neurobiology and Anatomy, Xuzhou Medical College, Xuzhou, Jiangsu, P.R. China

Abstract. – OBJECTIVE: We wished to test whether glial cell line-derived neurotrophic factor (GDNF) stimulates proliferation of gliomas by up-regulating expression of nuclear cyclins PCNA and Ki37.

MATERIALS AND METHODS: As a model, we tested rat C6 glioma cell line exposed to basal conditions, vehicle control, or exogenous GDNF at different concentrations (0-90 μg/L) or different times (0-72 hours). Cell proliferation was quantified by MTT test, cell cycle by flow cytometry and propidium iodide staining, expression of PCNA and Ki67 by intracellular antibody staining and flow cytometry.

RESULTS: We first observed that cell proliferation was most stimulated by GDNF at concentration of 70 μg/L and incubation time of 48 hours. Using this concentration and incubation time, we next documented that GDNF increased the percentage of cells in the S phase (47.98% vs. 32.57% in basal cells; $p < 0.05$), while not affecting the percentage of cells in G0/G1 or G2/M phases. Finally, we demonstrated that expression of both PCNA and Ki67 was significantly increased in cells exposed to GDNF.

CONCLUSIONS: We demonstrate that GDNF stimulates proliferation of glioma cells by up-regulating expression of cyclins PCNA and Ki-67.

Key Words:
Glioma, Glial cell line-derived neurotrophic factor, Cell proliferation, Cell cycle, PCNA, Ki67.

Introduction

The glial cell line-derived neurotrophic factor (GDNF) was separated and purified from rat glial cell line B49[1]. GDNF was initially thought to promote survival and differentiation of embryonic dopaminergic neurons[2,3]. Further studies, however, revealed that GDNF also plays an important role in proliferation of gliomas, which are one of the most common types of intracranial tumors[4]. Thus, antisense oligonucleotides against GDNF significantly reduce proliferation of rat C6 glioma cells[5,6]. It was also shown that GDNF promotes glioma cell proliferation through RAC-alpha serine/threonine kinases (AKT) and c-Jun N-terminal kinase (JNK)[7,8].

The goal of our study was to further examine the mechanisms of GDNF-driven proliferation of glioma cells. Specifically, we tested potential effects of GDNF on expression of two cyclins, proliferating cell nuclear antigen (PCNA) and Ki-67. PCNA, an acidic nuclear protein with a molecular weight of 36 kDa, is expressed in proliferating cells. This protein is essential to DNA synthesis. PCNA expression positively correlates with pathological grade of gliomas[9]. Ki-67 is a nuclear antigen in proliferating cells. It is a marker of cell proliferative activity with a yet unclear function. Immune response induced by Ki-67 is closely related to cell cycle[10]. Ki-67 appears to be one of the most reliable indicators of cancer cell proliferation. Ki67 is overexpressed in malignant gliomas[11] and is related to the development, metastasis, and prognosis of this tumor[12]. Here we present evidence that GDNF stimulation of C6 glioma cell proliferation is associated with up-regulation of both cyclins PCNA and Ki67.

Materials and Methods

Reagents

Dulbecco's modified Eagle's medium (DMEM) and newborn calf serum were purchased from Gibco (Grand Island, NY, USA). Trypsin was from Difco (Los Angeles, CA, USA); GDNF (dissolved in 0.1% bovine serum albumin: BSA) was from Sigma-Aldrich (San

Corresponding Author: Dianshuai Gao, MD; e-mail: gds@xzmc.edu.cn

Francisco, CA, USA). The methyl thiazolyl tetrazolium (MTT) and propidium iodide (PI) kits were, respectively, from Beyotime Institute of Biotechnology (Zhen Jiang, China) and GenMed (Shang Hai, China). Anti-PCNA (FITC conjugate), anti-Ki67 (PE conjuage), and isotype control antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Culture of C6 Rat Glioma Cell Line

This cell line was procured as a kind gift from the School of Medicine at Zhejiang University (Hang Zhou, China). The cells were cultured in DMEM supplemented with 10% newborn calf serum. The medium was changed every 1-2 days. The cells were lifted with 0.25% trypsin every 3-4 days, counted, and plated.

Cell Proliferation Assay

Cells (seeding concentration of 2×10^7/L) were grown on 96-well culture plates (100 µl/well) and exposed to basal conditions (serum-free DMEM), vehicle control (DMEM supplemented with BSA), or different doses of GDNF (10, 30, 50, 70, or 90 µg/L). In addition to testing dose response, we also assessed the effects of exposure to GDNF for different incubation times (12, 24, 48, or 72 hours) on glioma cell proliferation.

After exposure to experimental conditions, 10 µl of a 5 g/L MTT solution were added to each well, and cells were cultured for 4 hours. Then, 100 µl of dimethyl sulfoxide (DMSO) was added to each well, and cells were incubated for further 4-6 hours. After MTT crystals were completely dissolved, optical densities were measured at 570 nm. All experiments were repeated three times.

Cell Cycle Assay

Cells were collected by centrifugation and washed twice with cold phosphate-buffered saline (PBS). Then, cold 70% ethanol was added, and cells were fixed at 4°C overnight. Cells were pelleted by centrifugation, washed with 1 ml of PBS, and resuspended in 500 µl of propidium iodide (PI) solution. The cells were incubated with PI in the dark at 4°C for 30 min. The cell cycle distribution was measured by flow cytometry.

Expression of Cyclin Expression by Flow Cytometry

Cells were collected by centrifugation, washed twice with cold PBS, fixed in 1 ml of 4% formaldehyde for 40 min, and re-centrifuged. The supernatants were removed, and cells were washed with PBS. Then, cells were resuspended with 1 ml of Permeabilization Buffer and incubated at 4°C overnight. The next day, cells were collected by centrifugation and stained with primary antibody in the dark at 4°C for 1 hour. Afterwards, primary antibody was removed, and cells were washed twice with PBS. Cyclin expression was quantified by flow cytometry.

Statistical Analysis

The cell proliferation rate was calculated as follows: cell proliferation rate = (optical density$_{\text{experimental group}}$ − optical density$_{\text{control group}}$) / optical density$_{\text{experimental group}}$ × 100%. For other statistical analyses, we used SigmaStat (Irsoftware, Shanghai, China). Experimental data were expressed as mean ± SD. Comparisons between treatment groups were done using Dunnett or *Q* tests. The p value of < 0.05 was considered statistically significant.

Results

Optimal Dose and Exposure Time for GDNF to Promote Proliferation of Rat C6 Cells

The MTT assay was used to quantify on proliferation of rat C6 cells exposed to GDNF added at different concentrations and for different periods of time. As shown in Table I, cell proliferation significantly increased on incubation with GDNF at concentrations of ≥ 50 µg/L and incubation times of ≥ 24 hours. The maximal increase of cell proliferation was achieved using a concentration of 70 µg/L and incubation time of 48 hours (Table I). Therefore, these concentration and incubation time were used in subsequent experiments.

Effects of GDNF on Cell Cycle Phases

The effects of GDNF on cell cycle in rat C6 cells were documented using flow cytometry (Figure 1). We documented that proliferation of C6 cells was associated with increased percentage of cells in the S phase (47.98% vs. 32.57% in basal cells; $p < 0.05$). In contrast, the percentage of cells in G_0/G_1 or G_2/M phases was not significantly different between basal and GDNF-stimulated cells. We concluded that the effects of GDNF on proliferation of C6 glioma cells mainly occur during the S-phase of cell cycle.

Table I. Effects of different concentrations of GDNF and different exposure times on proliferation of C6 cells.

Experimental conditions	Incubation time, hours							
	12		24		48		72	
	Optical density	Proliferation rate %	Optical density	Proliferation rate %	Optical density	Proliferation rate %	Optical density	Proliferation rate %
Basal cells	0.117 ± 0.021	0	0.153 ± 0.022	0	0.182 ± 0.009	0	0.167 ± 0.031	0
Vehicle control	0.109 ± 0.018	0	0.144 ± 0.032	0	0.163 ± 0.033	0	0.154 ± 0.024	0
GDNF, 10 µg/L	0.119 ± 0.029	1.68	0.156 ± 0.041	1.92	0.191 ± 0.023	4.71	0.172 ± 0.045	2.91
GDNF, 30 µg/L)	0.121 ± 0.032	3.31	0.169 ± 0.027	9.47	0.209 ± 0.022	12.92	0.189 ± 0.015	11.64
GDNF, 50 µg/L	0.122 ± 0.015	4.10	0.194 ± 0.039*	21.13	0.259 ± 0.019*	29.73	0.235 ± 0.029*	28.94
GDNF, 70 µg/L	0.125 ± 0.031	6.40	0.246 ± 0.021*,#	37.80	0.418 ± 0.032*,#,&	56.46	0.292 ± 0.023*,#	42.81
GDNF, 90 µg/L	0.119 ± 0.024	1.68	0.219 ± 0.036*	30.14	0.297 ± 0.025*	38.72	0.264 ± 0.041*	36.74

Footnote: The effects of GDNF on C6 cells were quantified by MTT assay. Data are presented as mean ± SD of three experiments. *$p < 0.05$ vs. basal and vehicle control cells. #$p < 0.05$ vs. cells exposed to GDNF for the same amount of time but at different concentrations. &$p < 0.05$ vs. cells exposed to GDNF at the same concentration, but for different amounts of time.

Effects of GDNF on Expression of Cyclins PCNA and Ki67

We next quantified expression of two cyclins, PCNA and Ki67. Our studies demonstrated that percentage of cells expressing PCNA increased to 78.24% in cells stimulated with GDNF, which was a substantial increase over 28.97% in basal cells ($p < 0.05$; Figure 2). Similarly, percentage of cells positive for Ki67 increased from 5.52 under basal conditions to 20.78% in GDNF-stimulated cells ($p < 0.05$; Figure 3). These results indicated that GDNF stimulation of C6 glioma cell proliferation involves upregulation of expression of two cyclins, PCNA and Ki67, with both of them being highly expressed in the S phase.

Discussion

Gliomas are the most common type of intracranial cancer and account for 70% of primary central nervous system cancers. The patients are typically treated with a combination of radiotherapy and chemotherapy. Unfortunately, these treatments do not warrant a favorable prognosis. Here we tested the mechanisms of GDNF stimulation of glioma cell proliferation to reveal new targets for glioma treatment. We utilized C6 glioma cells which are obtained from Wistar rats and induced with N-nitrosomethylurea. These cells are commonly used in experimental researches on gliomas[13].

GDNF is a member of the GDNF family of ligands[14]. In brain gliomas, expressions of GDNF and its receptor GFR 1 are significantly higher than in normal brain tissue[15]. Specifically, GDNF content is approximately five times higher in glioma cells[15]. Abnormal expression of GDNF may explain increased glioma proliferation. GDNF modulates many biological reactions, such as cell proliferation, differentiation, apoptosis, and tumorigenesis. These effects involve Akt and JNK[7,8]. GDNF is a powerful stimulator of proliferation of glioma cells[16]. Specific mechanisms of this phenomenon are still unclear. In the present work, we tested the effects of exogenous GDNF on cultured rat C6 glioma cells.

In vivo, cell proliferation cycle is under strict regulation by cyclins. PCNA is an important regulatory factor of replication, providing a platform for the function of DNA pol δ/ε and other replication proteins. Importantly, DNA pol α can be transformed to a polymerase that catalyzes DNA

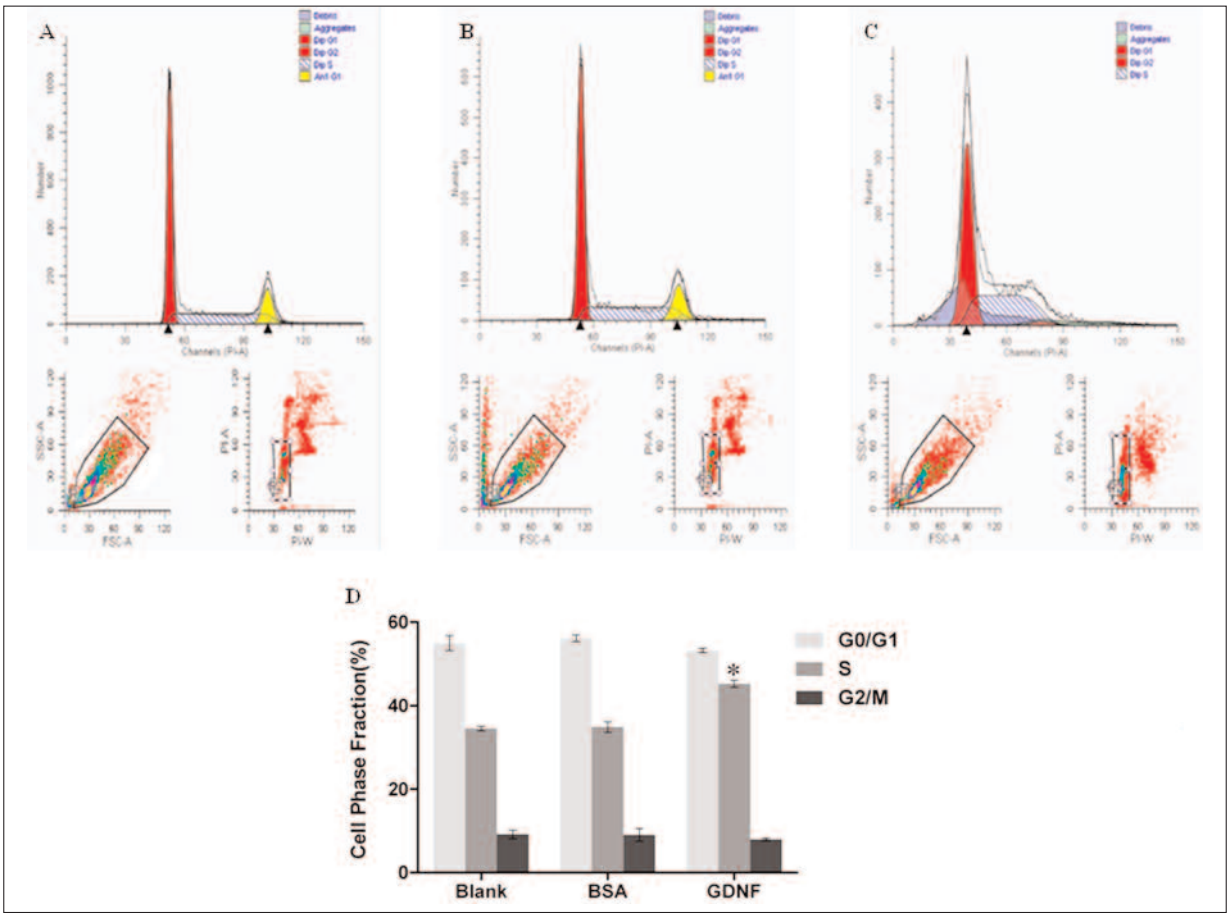

Figure 1. Effects of GDNF on cell cycle phases in rat C6 glioma cells. Effects of GDNF were measured by flow cytometry. ***A,-B,*** Respectively, representative images from cells under basal conditions and exposed to vehicle control. ***C,*** Image of cells exposed to 70 μg/L of GDNF for 48 hours. ***D,*** The above experimental data are presented as mean ± SD of three experiments. "Blank" = basal conditions and "BSA"= vehicle control; **p* < 0.05 vs. basal cells.

synthesis only when PCNA is present in the replication fork[17]. As a nucleoprotein, PCNA is required for DNA synthesis and participate in DNA reproduction. PCNA is an objective indicator of cell proliferative activity. Our findings demonstrate that GDNF stimulation of C6 glioma cell proliferation involves up-regulation of PCNA expression. A similar finding was true with regard to Ki-67, a nuclear protein expressed during G1, S, G2 and M phases of cell cycle and a marker of cell proliferation. GDNF treatment of glioma cells up-regulated Ki-67 expression in the S phase of cell cycle.

Conclusions

We demonstrate that GDNF stimulates proliferation of glioma cells by up-regulating expression of cyclins PCNA and Ki-67.

Acknowledgements

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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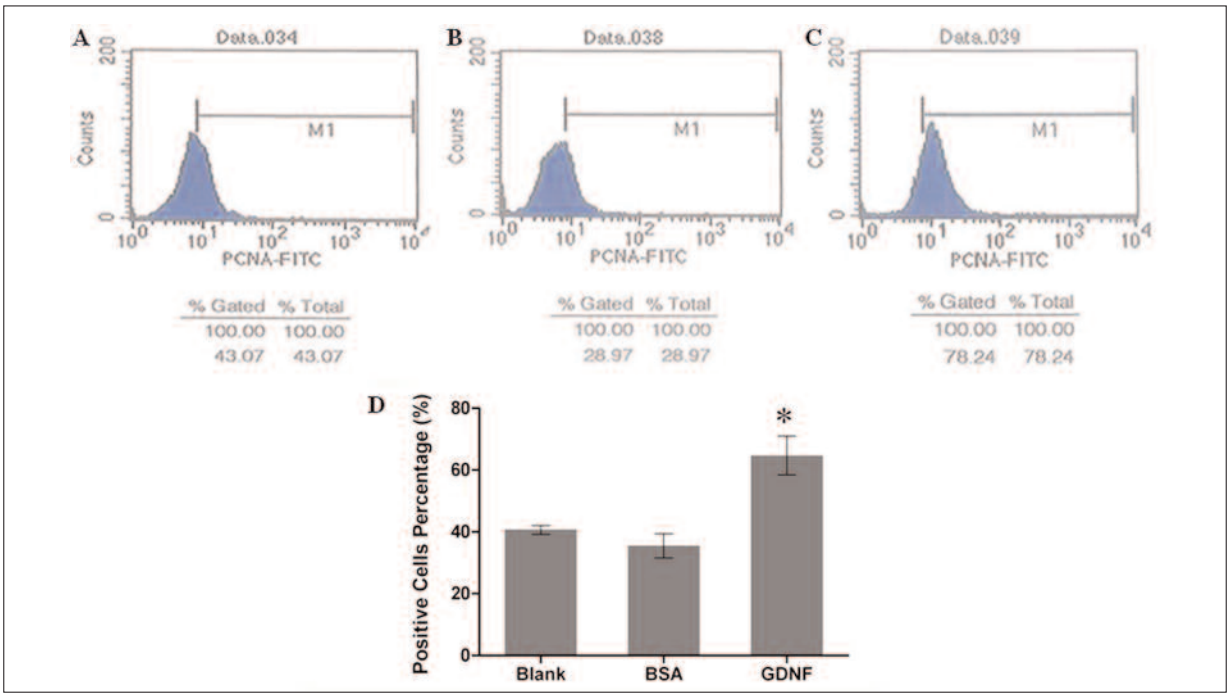

Figure 2. Effects of GDNF on expression of the cyclin PCNA in rat C6 glioma cells. Effects of GDNF were measured by flow cytometry. ***A,-B,*** Respectively, representative images from cells under basal conditions and exposed to vehicle control. ***C,*** Image of cells exposed to 70 μg/L of GDNF for 48 hours. ***D,*** The above experimental data are presented as mean ± SD of three experiments. "Blank" = basal conditions and "BSA"= vehicle control; *$p < 0.05$ vs. basal cells.

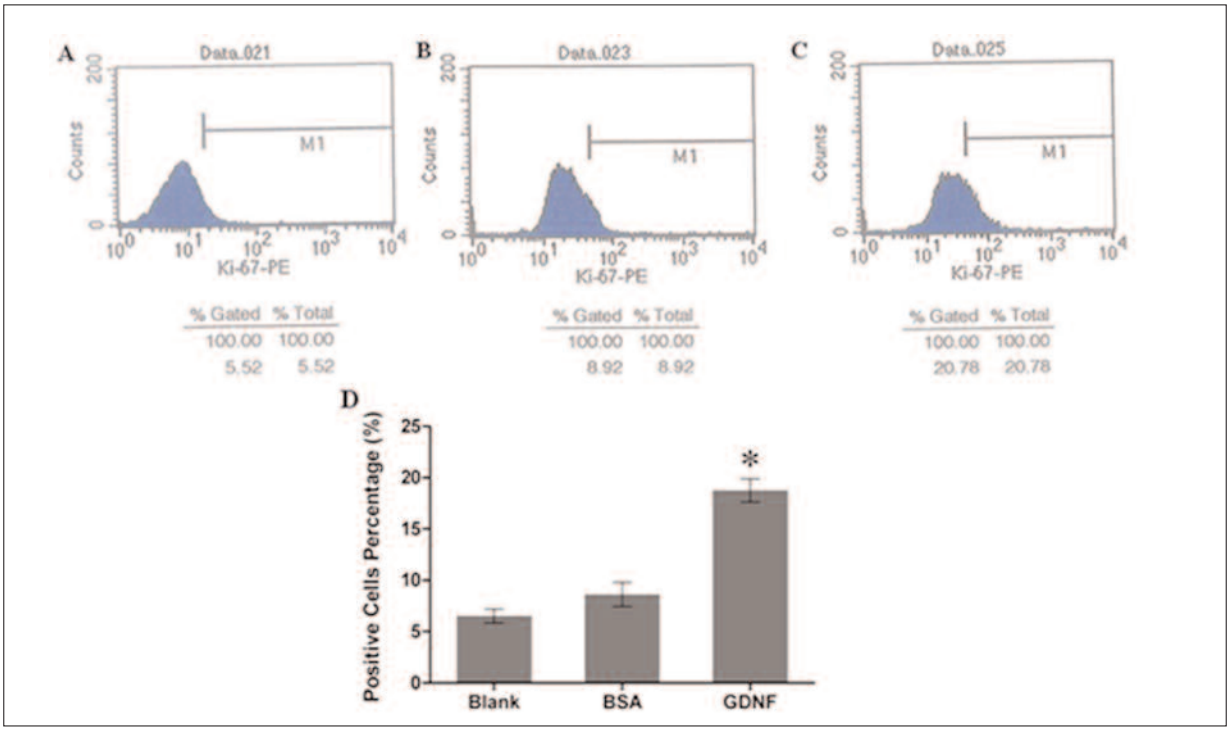

Figure 3. Effects of GDNF on expression of the cyclin Ki67 in rat C6 glioma cells. Effects of GDNF were measured by flow cytometry. ***A,-B,*** Respectively, representative images from cells under basal conditions and exposed to vehicle control. ***C,*** Image of cells exposed to 70 μg/L of GDNF for 48 hours. ***D,*** The above experimental data are presented as mean ± SD of three experiments. "Blank" = basal conditions and "BSA"= vehicle control; *$p < 0.05$ vs. basal cells.

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