Effect of Human Cytomegalovirus Infection on Nerve Growth Factor Expression in Human Glioma U251 Cells[1]

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Objective To explore the change of endogenic nerve growth factor (NGF) expression in human glioma cells infected with human cytomegalovirus (HCMV). **Methods** U251 cells were cultured in RPMI 1640 culture medium and infected with HCMV AD169 strain *in vitro* to establish a cell model of viral infection. Morphologic changes of U251 cells were observed under inverted microscope before and after infection with HCMV. Expression of NGF gene and protein of cells was detected by RT-PCR and Western blotting before and after infection with HCMV. **Results** The cytopathic effects of HCMV-infected cells appeared on day 5 after infection. However, differential NGF expression was evident on day 7. NGF expression was decreased significantly in U251 cells on day 7 after infection in comparison with control group ($P<0.05$). **Conclusion** HCMV can down-regulate endogenous NGF levels in human glioma cell line U251.

Key Words: Human cytomegalovirus; U251 cells; Nerve growth factor

INTRODUCTION

Human cytomegalovirus (HCMV), a member of the herpesvirus family, is the most common etiologic agent of congenital anomalies of the central nervous system (CNS) and causes intrauterine infection in humans, with an average incidence of approximately 1.0% among live births[1]. It is estimated that 5%-10% of embryos with congenital HCMV infection have severe neurologic damage at birth, such as microcephaly and perivascular calcification[2]. To date, the mechanism underlying the injuries induced by this kind of viral infection remains uncertain.

Nerve growth factor (NGF), a metabolically active peptide, was first isolated in nerve tissuek, and plays a key role in differentiation, development, and survival of sympathetic and sensory nerve cells, neural stem cells and cholinergic neurons of the CNS[3-4]. Apoptosis and cell death may be induced by NGF withdrawal from culture medium of nerve cells[5-7]. During the embryonic stage, NGF is mainly secreted by neuroglia cells, which are more susceptible to HCMV infection than other nerve cells[3]. In this study, we analyzed the gene expression of normal and infected human glioma cell lines by RT-PCR and Western blotting, showing that HCMV infection is associated with alterations in expression of NGF genes.

MATERIALS AND METHODS

Cell Cultures and Virus Infection

Human glioma cell line U251 (American Type Culture Collection, Manassas, VA, cell line number: 99522) was purchased from Shanghai Cell Resource Center of Chinese Academy of Sciences. U251 cells were propagated in RPMI 1640 (Gibco) medium with 10% fetal bovine serum (FBS, Gibco). Cells were maintained under subconfluent conditions at 37 ℃ in an atmosphere containing 5% (v/v) humidified CO_2.

HCMV AD169 (kindly provided by France Pasteur Laboratory and expanded in our laboratory) was titered by plaque titration in human embryonic lung fibroblast (HELF) cells and expressed as the

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number of plaque-forming units (PFU) per milliliter. U251 cells were infected with HCMV AD 169 strain at approximately 5 PFU per cell. Viral infections were performed in a serum-free medium containing 5% CO_2 for 1 h at 37 ℃. Virus was removed and the medium was replaced with RPMI 1640 containing 10% FBS and cells were cultured as described above.

Assays for HCMV Entry into U251 Cells

Six hours after infection, RT-PCR was performed to detect the expression of HCMV IE mRNA. Briefly, 1×10^6 cells were collected and total RNA was extracted with Trizol (Invitrogen). Reverse transcription was synthesized into cDNA with Oligo (dT), and the total volume was 20 μL containing 2 μL total RNA. Plasmid pRSV72 was diluted at 1:100 as a template for IE amplification PCR, which was taken as a positive control. Two primers of HCMV IE were designed according to the whole gene sequence of HCMV strain AD169 from GenBank (NC_001347). The primer pairs used were: IE forward (5'-CAAGAGAAAGATGGACCCTG-3') and reverse (5'-ACGAGTTCTGCCAGGACATC-3'); β-actin forward (5'-ACCGTGGAGAAGAGCTACGA-3') and reverse (5'-GTACTTGCGCTCAGAAGGAG-3'). Thirty cycles of PCR amplification were performed at 94 ℃ for 1 min, at 55 ℃ for 1 min, and 72 ℃ for 2 min. The expected size of PCR products for detecting IE and β-actin was 242 bp and 109 bp, respectively.

NGF RT-PCR

Total RNA was isolated from infected cells at various time pints post-infection using TRIzol (Invitrogen) according to its manufacturer's instructions. Complementary DNA (cDNA) was obtained by reverse transcription of 1 μg total RNA (Thermoscript; Life Technologies) using oligo$(dT)_{20}$ primer.

PCR was performed in a 10-μL volume. cDNA was added to the PCR mixture containing 0.5 U Taq polymerase (Eurogentec, Seraing, Belgium), 0.2 mmol/L dNTPs, 0.5 μmol/L specific primers and 1.5 mmol/L $MgCl_2$. RT without enzyme was taken as a negative control. PCR was carried out in a Perkin Elmer thermal cycler 9600 (Applied Biosystems, Lennik, Belgium). After denaturation at 95 ℃ for 1 min, 57 cycles (25 for β-actin and 32 for NGF) were performed at 94 ℃ for 10 s, at 60 ℃ for 10 s, at 72 ℃ for 30 s, and a final extension at 72 ℃ for 10 min.

Following NGF primers were used: forward (5'-CACACTGAGGTGCATAGCGT-3') and reverse (5′-TGATGACCGCTTGCTCCTGT-3'). The sequences of β-actin primers are 5'-ACCGTGGAGAAG-AGCTACGA-3' and 5'-GTACTTGCGCTCAGAAG-GAG-3'. cDNA was equalized for expression of the housekeeping gene β-actin. PCR products were electrophoresed on 1.5% agarose gel in a Tris-borate/EDTA electrophoresis buffer, stained with ethidium bromide, visualized by UV trans-illumination and analyzed by densitometry. Expected size of the amplified products was 352 bp for NGF and 109 bp for β-actin, respectively.

Indirect Immunofluorescence

After infection, cells were washed twice with PBS, fixed with 4% paraformaldehyde in PBS for 10 min, permeated in PBS containing 0.1% Triton X-100 and 5% bovine serum albumin for 30 min before detection of NGF with immunofluorescence. A medium containing anti-NGF rabbit polyclonal antibody (Beijing Boisynthesis Biotechnology Co., Ltd.) at a dilution of 1:200 was introduced into the culture chamber and cells were incubated overnight at 4℃. After washed three times with PBS, cells were stained with anti-rabbit FITC green-conjugated secondary antibody (Beijing Boisynthesis Biotechnology Co., Ltd.) at a dilution of 1:100 at 37 ℃ for 30 min. Cell images were subsequently captured under fluorescence microscope.

Protein Extraction and Western Blotting

Protein extract was obtained from U251 cells using RIPA lysis buffer (50 mmol/L Tris, 150 mmol/L NaCl, 1% TritonX-100, 1% sodium deoxycholate, 0.1% SDS) and phenylmethylsulfonyl fluoride (PMSF). After 30 min of incubation at 4 ℃, lysates were heated at 100 ℃ for 5 min and centrifuged at 12 000×g for 20 min at 4 ℃. Samples were run on 10% polyacrylamide gels. Proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Millipore), and blots were stained with amido black to ensure that each lane had an equivalent amount of protein. The membrane with transferred proteins was blocked with 5% dry unfit milk in TBS 1× containing 0.1% Tween 20 (TBST) for 1 h at room temperature, incubated with anti-NGF rabbit polyclonal antibody diluted at 1:200 in TBST for 2 h at room temperature and then with horseradish peroxidase-labeled secondary antibody for 1 h at room temperature. After washed in TBST, the membrane was developed using ECL reagents and assayed by Western blot for β-actin according to the protocol of Amersham. lysates. Protein bands were quantified using Quantity One software (BioRad). Paired mean comparisons were performed to assess concentration-dependent changes in the intensity of native protein bands.

Data Acquisition and Analysis

All experiments were performed in triplicate, and results are plotted as $\bar{x} \pm s$. The statistical significance between experimental means was determined with Student's t test, and $P<0.05$ were considered statistically significant.

RESULTS

Morphological Changes in Cells after Viral Infection

The morphology of glioma cells appeared to be grossly normal in the first 3 days after HCMV infection. On day 5, cytopathic effects, such as cellular swelling, cellular confluence, and presence of honeycombs, were evident in more than 50% cells. Seven days after infection, these cytopathic effects were observed in nearly 100% cells, with honeycomb-giant cells and detachment of some cells (Fig. 1).

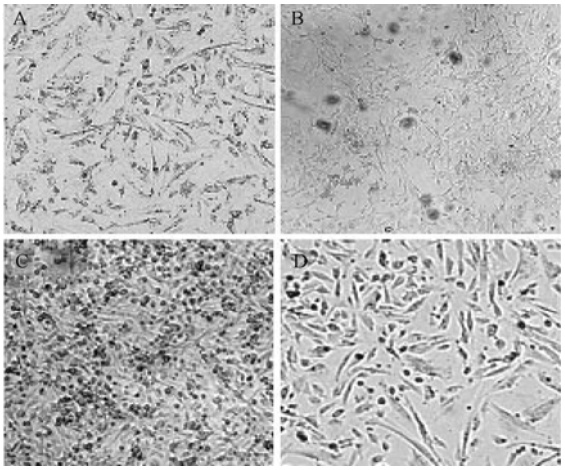

FIG. 1. Morphology of U251 cells 3 days (A), 5 days (B), 7 days (C), after HCMV infection (×100), and control cells (D).

Confirmation of HCMV Infection Model

Electrophoresis on 1.2% agarose gel showed a 242 bp IE fragment by RT-PCR amplification from infected cells (Fig. 2). RT-PCR showed that HCMV IE in human U251 cells was positive (lane 2) compared with the control (lane 3), demonstrating that the model of HCMV infection can be successfully established.

NGF mRNA Expression in U251 Cells

β-actin and NGF were expressed in U251 cells at different time points, and 110 bp and 350 bp objective gene fragments were obtained. Special amplification bands after agarose gel electrophoresis were in accordance with the expected fragment length (Fig. 3). RT-PCR showed no difference in expression levels of β-actin and NGF from day 1 to day 5 after infection. Student's t test displayed that the expression levels of β-actin and NGF were significantly lower on day 7 (0.521±0.053) than on day 0 (0.824±0.176, $P<0.05$). The NGF albumin level in control group did not show any significant change at different time points.

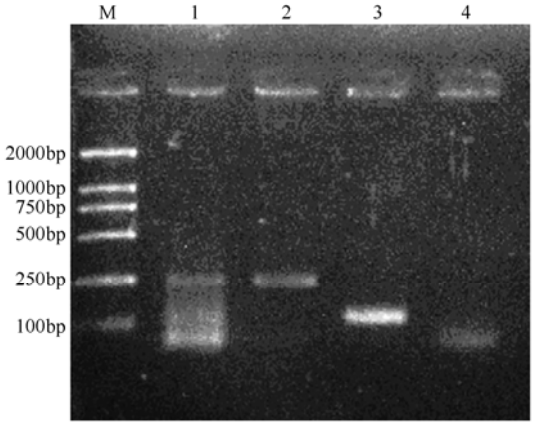

FIG. 2. RT-PCR showing HCMV expression in U251 cells 6 h after HCMV infection. M: DL2000 marker, 1: infected group, 2: positive control, 3: β-actin as an internal reference, 4: negative control.

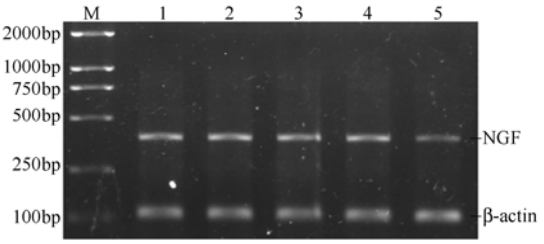

FIG. 3. Semi-quantitative RT-PCR analysis showing NGF mRNA expression in U251 cells. M: DL2000 marker, 1: control, 2-5: 1, 3, 5, 7 days after infection of U251 cells with HCMV.

Expression of NGF Protein

To further verify the results of PCR, indirect immunofluorescence was used to observe the changes of NGF protein expression in U251 cells infected with HCMV. The intensity of immunostaining for

NGF decreased on day 7 compared with the control (Fig. 4D). Western blotting revealed that the NGF protein expression was decreased in U251 cells infected with HCMV (Fig. 5). Significant decreases in the expression levels of NGF protein were observed using two immunomethods. However, compared with normal cells, NGF protein was expressed in cells infected with MCMV on day 7 when the cells were stained with immunocytochemistry.

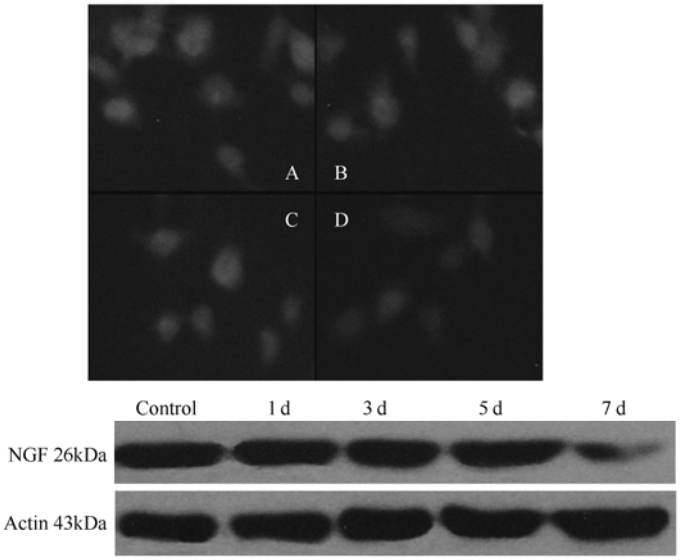

FIG. 4. Indirect immunofluorescence of NGF in uninfected (A) U251 cells, and 3 (B), 5 (C) and 7 (D) days after infection of U251cells with HCMV.

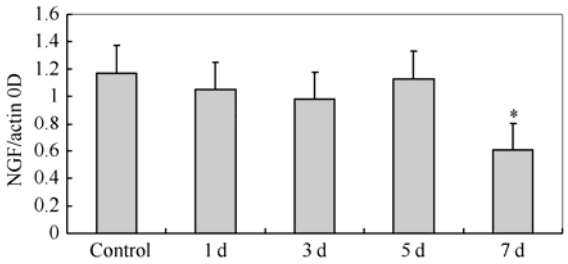

FIG. 5. Expression of NGF and β-actin. Proteins were extracted from normal control and infected U251 cells. Intensity of bands was analyzed with Quantity One Software. Protein expression level was presented as a percentage of control values obtained from Western blot analysis. $^{*}P<0.05$ *vs*. control group.

DISCUSSION

NGF, a neurotrophic factor is one of the most important bioactivators of the nervous system with extensive biological effect. NGF is mainly distributed in the hippocampus, cerebral cortex, striatum, cerebellum, neural stem cells (NSC) in lateral ventricle and dentate gyrus region of the hippocampus. NSC can self-renew and have multiplex differentiation potentials. Development of NSC includes multiplication, existence, movement, and differentiation. NGF plays an important role in regulation of NSC development. During the development of NSC, enormous neural progenitor cells will die[8]. NGF cannot directly promote the multiplication of NSC, but can maintain the survival and increase the number of neural progenitor cells[9-10], and maintain the normal functions of NSC, inducing their movement[11-12] and differentiation[3, 13]. NGF can promote the differentiation, maintain the growth and development, and improve the injury repair[14] of neurons, but the expression and

biological effects of NGF differ in different development periods of the nervous system. NGF in the central nervous system during embryonic period is mainly expressed by astrocytes[15-16], and mainly synthesized in nerve cells[17] of mature brain. During the embryonic period, NGF can maintain the survival of neurons, control the survival time and promote the differentiation of neurons. If ignatovich[18] is used to treat embryonic animals with NGF antibody, the neurons would decrease by 80%, and treatment of adult animals with NGF would not reduce the number of neurons, indicating that neurons depend greatly on NGF during the embryonic period.

HCMV is one of the main causes for congenital infection and fetal deformation[19]. However, its exact teratogenic mechanism is not clear. As a neurotropic virus, HCMV can infect various nerve cells, with astrocytes being most sensitive[20-21]. This research, for the first time, corfirmed that HCMV infection could induce abnormal NGF gene expression in U251 cells, indicating that NGF gene expression is significantly downregulated in U251 cells infected with HCMV.

In conlusion, HCMV downregulates the NGF expression, induces cell apoptosis reduces NGF density of nervous organization, causes abnormal development of NSC and neurons, and leads to deformation by infecting astrocytes during the embryonic period[16]. Regulation of NGF expression and biological effects is influenced by many other factors. The regulating function and mechanism of HCMV underlying NGF expression in nerve cells require further research.

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