

RESEARCH COMMUNICATION

Induction of Apoptosis in Glioma Cells and Upregulation of Fas Expression Using the Human Interferon- β Gene

Yan Guo, Gan Wang, Wen-Wei Gao, Shi-Wen Cheng, Ren Wang, Shi-Ming Ju, He-Li Cao, Heng-li Tian*

Abstract

We investigated whether IFN- β inhibits the growth of human malignant glioma and induces glioma cell apoptosis using the human IFN- β gene transfected into glioma cells. A eukaryonic expression vector (pSV2IFN β) for IFN- β was transfected into the glioma cell line SHG44 using liposome transfection. Stable transfection and IFN- β expression were confirmed using an enzyme-linked immunosorbent assay (ELISA). Cell apoptosis was also assessed by Hoechst staining and electron microscopy. *In vivo* experiments were used to establish a SHG44 glioma model in nude mice. Liposomes containing the human IFN- β gene were injected into the SHG44 glioma of nude mice to observe glioma growth and calculate tumor size. Fas expression was evaluated using immunohistochemistry. The IFN- β gene was successfully transfected and expressed in the SHG44 glioma cells *in vitro*. A significant difference in the number of apoptotic cells was observed between transfected and non-transfected cells. Glioma growth in nude mice was inhibited *in vivo*, with significant induction of apoptosis. Fas expression was also elevated. The IFN- β gene induces apoptosis in glioma cells, possibly through upregulation of Fas. The IFN- β gene modulation in the Fas pathway and apoptosis in glioma cells may be important for the treatment of gliomas.

Keywords: Interferon- β - human malignant glioma - apoptosis - therapy

Asian Pacific J Cancer Prev, 13, 2837-2840

Introduction

At present, there is no effective treatment for patients with malignant and recurrent glioma. Patients with malignant glioma have very poor prognosis, even with surgical resection, radiation, and chemotherapy. Gene therapy has received particular attention as a promising treatment for malignant glioma.

IFNs are a family of natural glycoproteins that consist of IFN- α , β , and γ . The antiviral activity of IFNs led to their discovery. However, later data revealed that they also control cell growth and differentiation (Hertzog et al., 1994), inhibit oncogene expression, and activate T lymphocytes, natural killer cells, and macrophages. Interferon inhibits the growth of tumor blood vessels and cellular immunity activation (Nakahara et al., 2003; Konjević et al., 2010; Williams et al., 2010).

The efficacy of IFN therapy for various malignancies has been investigated for many years. Extensive clinical trials concluded that IFNs could be effective against many neoplasms. IFN- α is a rapid and potent inducer of apoptosis in H9 and U-266 cells, hematopoietic cell lines (Sangfelt et al., 1997). Human IFN- β has the strongest antiproliferative activity against human melanoma cell

lines. Histologic analysis of the injected nodules revealed that the IFN- β gene transfection induced apoptosis in human melanoma cells. These data suggest that the transfection of the IFN- β gene using cationic liposomes is a promising candidate for gene therapy for human melanoma (Kageshita et al., 2001). Continuous incubation of different human carcinoma cells with noncytostatic concentrations of IFN- α or IFN- β downregulates the transcription and protein production of basic fibroblast growth factor (Singh et al., 1995; Dinney et al., 1998), interleukin 8 (Oliveira et al., 1992), and collagenase type IV (Fabra et al., 1992; Gohji et al., 1994).

Furthermore, the mechanisms related to the inhibition of human malignant glioma growth by interferon- β remains unclear. Fas is a transmembrane protein that belongs to the TNF receptor family. After binding to the Fas ligand (FasL), Fas initiates an intracellular cascade that leads to the induction of apoptosis in target cells. The objective of this paper is to investigate whether IFN- β inhibits the growth of human malignant glioma and might be able to induce apoptosis on transfection of the IFN- β gene into glioma cells. We additionally investigated the role of IFN- β in apoptosis and modulation of the Fas pathway.

Department of Neurosurgery, the 6th Hospital Affiliated to Shanghai Jiaotong University, Shanghai, China *For correspondence: tianhengli@yeah.net

Materials and Methods

Animals

Female nude mice 6-8 weeks old were kept and bred under pathogen-free conditions in the animal facility of the Fourth Military Medical University. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the 6th Hospital Affiliated to Shanghai Jiaotong University.

Cell transfection

SHG44 Glioma Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 0.238% N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acids (HEPES), 100 U/mL penicillin, and 0.1 mg/mL streptomycin.

To prepare pSV2IFN β -containing liposomes, TMAG, DLPC, and DOPE at a molar ratio of 1:2:2 were dissolved in 0.5 mL of chloroform. The solvent was evaporated and the lipid film was wetted with 0.2 mL of phosphate-buffered saline (PBS) containing 20 μ g of pSV2IFN β (from Prof. J. Yoshida, Nagoya University, Japan) and then suspended with a vortex agitator for 2 min. The volume of the suspension was adjusted to 0.5 mL with PBS.

Aliquots of SHG44 cells (5×10^4 cells) were inoculated into each well of a Falcon plate with 1.5 mL of medium and incubated at 37 °C for 24 h under a humidified 5% CO₂ atmosphere. Then, pSV2IFN β (15 nmol of lipid/0.3 μ g of DNA/mL) was added to the medium and incubated for up to 6 days. The culture medium was collected on days 2, 4, and 6 after the addition of the liposomes. The IFN- β concentration in the medium was measured using an ELISA. The growth-inhibiting effect of the human IFN- β gene on the SHG44 glioma cells was also evaluated.

Detection of apoptosis

After 24 h of incubation with IFN- β , the cultured SHG44 cells were rinsed twice with PBS and fixed with methanol and glacial acetic acid (3:1) for 15 min at room temperature. After the fixative was aspirated, the culture plates were flooded with 10 mg/mL of Hoechst 33258 (Beyotime Biotechnology Inc., Nantong, China) in PBS and incubated in the dark for 15 min. The cells were then rinsed thrice with PBS and cover-slipped with 0.1 M of n-propyl gallate in 80% glycerol. Then, the cells were examined using a fluorescent microscope with a DAPI filter. The SHG44 glioma cells were observed using a transmission electron microscope according to standard methods.

Gene transfer in vivo growth

The SHG44 glioma cells were collected, washed twice in serum-free DMEM, and resuspended at 3×10^7 cells/mL for subcutaneous inoculation. Then, 100 μ L of DMEM containing 3×10^6 SHG44 cells was injected subcutaneously into the hind limbs of the nude mice. After 2 weeks, the mice were randomly assigned into the

following three treatment groups: group 1, intratumoral PBS injection (15 μ L); group 2, intratumoral injection of empty liposomes (150 nmol lipid in 15 μ L); and group 3, intratumoral injection of liposomes containing pSV2IFN β (3 μ g DNA and 150 nmol lipid in 15 μ L). The injections were performed every 3 days. Tumor volume was calculated using the formula $V(\text{cm}^3) = \pi D^3/6$, where D is the mean of the longest and shortest diameters of the glioma.

Histopathologic analysis

The tumors were excised from the nude mice 5 weeks after the administration of the treatments. The tumor tissues were fixed in 10% neutral buffered formalin, routinely processed, and embedded in paraffin. Histopathologic analysis was evaluated using conventional hematoxylin and eosin staining. The detection of apoptotic cells in tissue sections was performed using a TdT-mediated dUTP nick end labeling (TUNEL) assay kit (Boshide Biotechnology Inc., Wuhan, China) according to the manufacturer's instructions. Fas expression in the glioma tissues was determined through immunohistochemistry staining according to the manufacturer's instructions (Boshide Biotechnology Inc., Wuhan, China).

Results

Human IFN- β production

Human IFN- β production was detected in the culture medium of the transfected SHG44 cells at 2, 4, and 6 days after the transfection. However, it was not found when PBS, empty liposomes, or pSV2IFN β were added to the cells. The amount of IFN- β with lip (pSV2IFN β) was 28.5 ± 5.6 , 53.2 ± 4.1 , and 63.4 ± 5.3 at 2, 4, and 6 days after the transfection. In this case, 1 mL of the SHG44 cell suspension in the culture medium was placed in each well. The culture medium was collected at 2, 4, and 6 days after transfection. The amount of IFN- β was measured using an ELISA (n = 5). The values given are means \pm SD.

Cell growth inhibition

The cultured SHG44 glioma cells were counted at 2, 4, and 6 days after the transfection with lip. The number of SHG44 glioma cells with PBS was 5.8 ± 0.6 , 12.4 ± 1.3 , and 19.4 ± 0.5 at 2, 4, and 6 days after the transfection. The number of SHG44 glioma cells with empty liposomes was 6.5 ± 0.9 , 10.2 ± 0.8 , and 13.1 ± 0.3 . The number of SHG44 glioma cells with lip (pSV2IFN β) was 6.2 ± 0.7 , 3.1 ± 0.3 , and 1.4 ± 0.5 . In this case, 1 mL of the glioma SHG44 cell suspension in the culture medium was placed in each well. Cell growth was evaluated on days 2, 4, and 6 by counting the number of trypan blue-excluding cells using a hemocytometer (n = 5). The values given are the means \pm SD expressed as the number of cells ($\times 10^4$).

Lip (pSV2IFN β) significantly suppressed the growth of SHG44 glioma cells. By contrast, PBS, empty liposomes, and pSV2IFN β did not significantly reduce cell growth.

Apoptosis in the cultured SHG44 cells

In the Hoechst staining, the SHG44 glioma cells in the control groups have normal morphologies. Nuclear

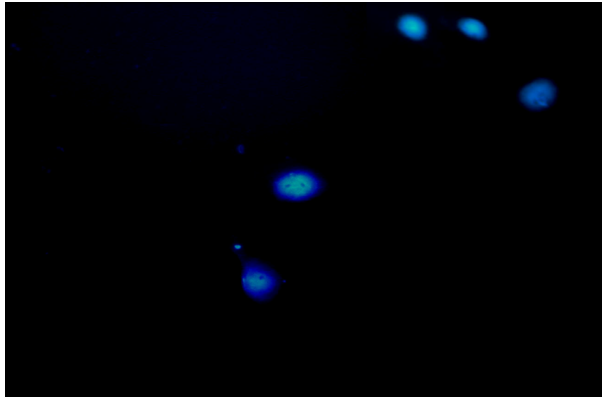


Figure 1. Nuclear Fragmentation Seen in Glioma SHG44 Cells ($\times 400$)

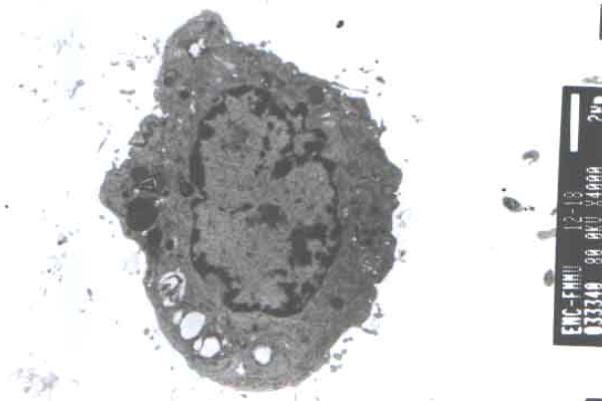


Figure 2. Nuclear Fragmentation Observed in a Glioma SHG44 Cell ($\times 4000$)

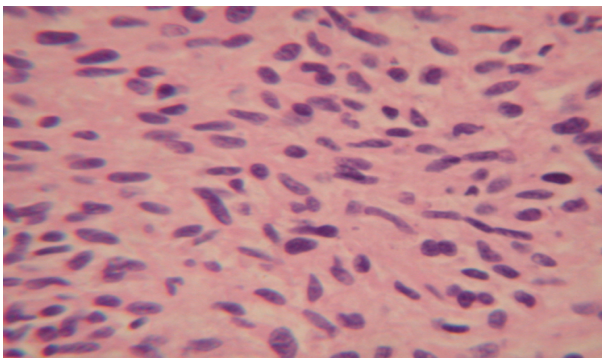


Figure 3. Haematoxylin and Eosin Stained of Glioma SHG44 Cells of Nude Mice ($\times 400$)

fragmentation was observed in the SHG44 glioma cells 48 h after IFN- β gene transfection (Figure 1). Characteristic apoptotic changes were observed in the SHG44 glioma cells under electron microscopy. Chromatin condensation was observed as sharply defined electron dense caps and the nuclear membrane and organelles were preserved (Figure 2).

Growth inhibition of glioma tumors

Tumors injected with PBS (Group 1) grew rapidly for 20 days from the time of injection. By contrast, Group 3, those with tumors injected with liposomes pSV2IFN β , suppressed tumor growth. However, the tumors did not disappear completely. No growth inhibition was observed in Group 2, those with tumors injected with empty liposomes. Human IFN- β was detected in Group 3. The mean tumor volumes in groups 1, 2, and 3 were 8.043

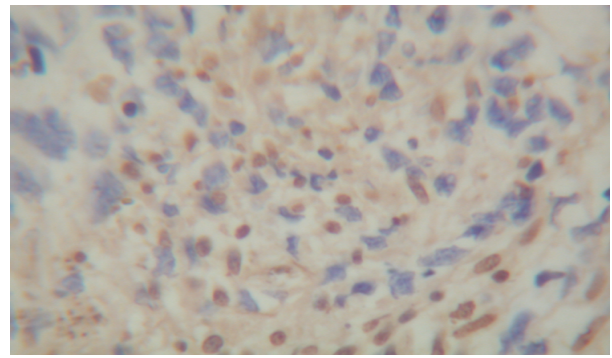


Figure 4. Apoptotic SHG44 Cells of Nude Mice were Detected Immunohistochemically Using A TUNEL Assay ($\times 400$)

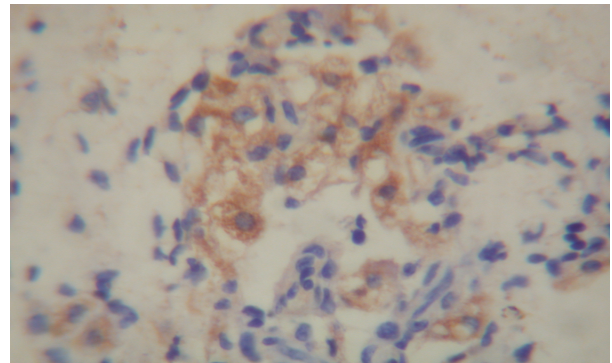


Figure 5. The Expression of Fas was Detected by Immunohistochemistry ($\times 400$)

$\text{cm}^3 \pm 1.68 \text{ cm}^3$, $7.918 \text{ cm}^3 \pm 1.76 \text{ cm}^3$, and $4.326 \text{ cm}^3 \pm 1.52 \text{ cm}^3$, respectively.

Histopathologic analysis

Histopathologic analysis of the hematoxylin and eosin-stained tumor sections was performed 5 weeks after implantation (Figure 3). Apoptotic SHG44 cells were immunohistochemically detected using a TUNEL assay kit (Figure 4). Fas expression was detected using an immunohistochemistry kit (Figure 5).

Discussion

In recent studies, IFN- β has multiple biological actions such as the modulation of gene expression and their protein products, immunomodulation, slowing of cell proliferation, and alteration of cell differentiation. Despite the clinical activity of IFN- β on malignant gliomas (Lin et al., 2004; Yoshida et al., 2004; Ito et al., 2010), its antitumor mechanism *in vivo* remains unclear.

The role of IFN- β in apoptosis and modulation of the Fas pathway was investigated.

The cultured SHG44 human glioma cells transfected with pSV2IFN β produced human IFN- β and their growth was inhibited *in vitro*. In the Hoechst staining, the SHG44 glioma cells in the control groups had normal cell morphologies. Nuclear fragmentation was observed in the glioma SHG44 cells at 48 h after IFN- β gene transfection. The characteristic apoptotic cell changes were observed in the SHG44 glioma cell under electron microscopy. In this study, human glioma cells transplanted subcutaneously into nude mice were clearly suppressed by the injection of

pSV2IFN β -containing liposomes. Immunohistochemical analysis revealed that the human IFN- β gene induced a significantly higher degree of apoptosis, which was responsible for the growth inhibition. Although the addition of exogenous human IFN- β has shown anti-proliferative effects against the glioma cell in vitro, higher local concentrations of human IFN- β continuously secreted by the transfected glioma cells may be more effective in vitro and in vivo. A 40-fold increase in the human IFN- β concentration is needed to obtain an inhibitory effect similar to that observed in human IFN- β gene transfection (Mizuno et al., 1990). The mechanism by which IFN- β gene therapy increases tumor cell apoptosis is unknown. Apoptosis is induced by IFN- γ in colorectal adenocarcinoma through the upregulation of Fas and Bax antigens and downregulation of Bcl-2 (Koshiji et al., 1998). In addition, IFN- α induces the regression of basal cell carcinoma by apoptosis induction via CD95 (Apo-1/Fas)-CD95 ligand interaction (Buechner et al., 1997). The upregulation of Fas expression in glioma tissues may contribute to the apoptotic activity of glioma cells. Intravenous human IFN- β administration is clinically effective against malignant gliomas. The human IFN- β can act as a sensitizer, enhancing toxicity against gliomas when given in combination with chemotherapeutic agents such as ACNU and MCNU (Wakabayashi et al., 2000).

In conclusion, IFN- β gene therapy is an effective treatment for malignant glioma and the high degree of apoptosis in glioma cells is responsible for the growth inhibition. Fas expression in malignant glioma in vivo was enhanced by IFN- β . Fas-mediated cytotoxicity was probably augmented by IFN- β . The upregulation of Fas prior to cell death suggests that the Fas pathway may be involved in this process although its exact role is still unclear. The mechanism by which IFN- β gene therapy increases tumor cell apoptosis is complicated and remains unclear. Further experimentation is required to evaluate Fas-susceptibility by sensitivity enhancers such as IFN- β .

Acknowledgements

The authors thank Prof. Heng-Li Tian for editorial support.

References

- Buechner SA, Wernli M, Harr T, et al (1997). Regression of basal cell carcinoma by intralesional interferon-alpha treatment is mediated by CD95 (Apo-1/Fas)-CD95 ligand-induced suicide. *J Clin Invest*, **100**, 2691-6.
- Dinney CP, Bielenberg DR, Perrotte P, et al (1998). Inhibition of basic fibroblast growth factor expression, angiogenesis, and growth of human bladder carcinoma in mice by mice by systemic interferon-a administration. *Cancer Res*, **58**, 808-14.
- Fabra A, Nakajima M, Bucana CD, Fidler IJ (1992). Modulation of the invasive phenotype of human colon carcinoma cells by organ-specific fibroblasts of nude mice. *Differentiation*, **52**, 101-10.
- Gohji K, Fidler IJ, Tsan R, et al (1994). Human recombinant interferons-b and g decrease gelatinase production and invasion by human KG-2 renal carcinoma cells. *Int J Cancer*, **58**, 380-4.
- Hertzog PJ, Hwang SY, Kola I (1994). Role of interferons in the regulation of cell proliferation, differentiation, and development. *Mol Reprod Dev*, **39**, 226-32.
- Ito S, Natsume A, Shimato S, et al (2010). Human neural stem cells transduced with IFN-beta and cytosine deaminase genes intensify bystander effect in experimental glioma. *Cancer Gene Ther*, **17**, 299-306.
- Kageshita T, Mizuno M, Ono T, et al (2001). Growth inhibition of human malignant melanoma transfected with the human interferon-beta gene by means of cationic liposomes. *Melanoma Res*, **11**, 337-42.
- Konjević G, Mirjačić Martinović K, Vuletić A, Babović N (2010). In-vitro IL-2 or IFN- α -induced NKG2D and CD161 NK cell receptor expression indicates novel aspects of NK cell activation in metastatic melanoma patients. *Melanoma Res*, **20**, 459-67.
- Koshiji M, Adachi Y, Sogo S, et al (1998). Apoptosis of colorectal adenocarcinoma (COLO 201) by tumour necrosis factor-alpha (TNF-alpha) and/or interferon-gamma (IFN-gamma), resulting from down-modulation of Bcl-2 expression. *Clin Exp Immunol*, **111**, 211-8.
- Lin W, Zhang X, Wang ZX, et al (2004). Growth inhibition of glioma cell line SHG44 by transfection of human interferon-beta gene liposome. *Chin J Neurosurg Dis Res*, **3**, 226-9.
- Mizuno M, Yoshida J, Sugita K, et al (1990). Growth inhibition of glioma cells transfected with the human beta-interferon gene by liposomes coupled with a monoclonal antibody. *Cancer Res*, **50**, 7826-9.
- Nakahara N, Pollack IF, Storkus WJ, et al (2003). Effective induction of antiglioma cytotoxic T cells by coadministration of interferon-beta gene vector and dendritic cells. *Cancer Gene Ther*, **10**, 549-58.
- Oliveira IC, Scivolino PJ, Lee TH (1992). Down regulation of interleukin 8 gene expression in human fibroblasts, unique mechanism of transcriptional inhibition by interferon. *Natl Acad Sci USA*, **89**, 9049-53.
- Sangfelt O, Erickson S, Castro J, et al (1997). Induction of apoptosis and inhibition of cell growth are independent responses to interferon-alpha in hematopoietic cell lines. *Cell Growth Differ*, **8**, 343-52.
- Singh RK, Gutman M, Bucana CD, et al (1995). Interferons alpha and beta down regulate the expression of basic fibroblast growth factor in human carcinomas. *Proc Natl Acad*, **92**, 4562-6.
- Wakabayashi T, Kajita Y, Hatano N, et al (2000). Clinicopathological study of oligodendroglial tumors, the effectiveness of interferon beta, ACNU/MCNU, and radiation (IAR/IMR) for anaplastic tumors. *Brain Tumor Pathol*, **17**, 29-33.
- Williams RF, Myers AL, Sims TL, Ng CY, Nathwani AC, Davidoff AM (2010). Targeting multiple angiogenic pathways for the treatment of neuroblastoma. *J Pediatr Surg*, **45**, 1103-9.
- Yoshida J, Mizuno M, Wakabayashi T (2004). Interferon-beta gene therapy for cancer, basic research to clinical application. *Cancer Sci*, **95**, 858-65.