Efficient Inhibition of In Vivo Human Malignant Glioma Growth and Angiogenesis by Interferon-β Treatment at Early Stage of Tumor Development

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ABSTRACT

Malignant gliomas are highly angiogenic and aggressive tumors. IFN-β has been used for the treatment of patients with malignant glioma; however, its antitumor mechanism in vivo remains unclear. To understand the in vivo antitumor effect and mechanism of recombinant human IFN-β (rhIFN-β) depending on the stages of tumor development or progression, we used orthotopic xenograft brain tumors generated by stereotactic intracerebral implantation of U-87 human glioma cells in nude mice. Mice bearing tumors 7 days (group 1) and 21 days (group 2) postimplant were treated with 2 × 10^8 IU/day of rhIFN-β or saline i.p. for 15 days, respectively. Tumor growth was suppressed by 69.6% in group 1 and 10.8% in group 2 compared with tumors of each control group treated with saline. rhIFN-β-treated group 1 animals showed 38% reduction in vascularization along with a 2.5-fold increase of the apoptotic index and no change in the proliferative index as compared with untreated tumors. The expression level of vascular endothelial cell growth factor and basic fibroblast growth factor was not affected by rhIFN-β treatment. rhIFN-β showed inhibitory activity on proliferation of U-87 cells, human umbilical vein endothelial cells, and PAM 212 murine keratinocytes in vitro. Our results indicate that the in vivo antitumor effect of rhIFN-β on malignant gliomas may be mediated, at least in part, via angiogenesis inhibition rather than antiprolifera-

tive activity and that rhIFN-β may be more effective for the treatment of malignant glioma patients at an early stage with minimal or microscopic tumor burdens rather than at an advanced stage of tumor development.

INTRODUCTION

Malignant gliomas, the most common primary brain tumors, are very aggressive tumors with a dismal prognosis despite advances in surgery, radiation therapy, and chemotherapy (1). Recent experimental evidence indicates that angiogenesis is an essential biological event encountered in tumor growth and progression, and inhibition of tumor-related angiogenesis may provide an alternative approach for tumor therapy (2). Malignant gliomas are characterized by rapid cell proliferation activity, high invasiveness into the surrounding brain, and increased degree of vascularity (1, 3). Morphological features of dense vascularity and endothelial cell proliferation of malignant gliomas are readily recognizable indications that distinguish them from other brain tumors, i.e., astrocytomas. Recent studies showed that tumor-related angiogenesis contributes significantly to the malignant phenotype of tumors (4). Blood-brain barrier or blood-tumor barrier, which hinders drug delivery into brain tissue, is considered to be one of the main problems associated with chemotherapy of brain tumors (1, 5). Therefore, an antiangiogenic approach targeting endothelial cells rather than tumor cells may be especially suitable for the treatment of malignant brain tumors. Several angiogenesis inhibitors were reported to induce regression or dormancy in a variety of primary and metastatic tumors (6–13), and we also previously reported that recombinant kringles 1–3 of human plasminogen effectively inhibit angiogenesis and growth of intracerebral malignant glioma in nude mice (11). Neither the antitumor mechanism nor the effective therapeutic guidelines of angiogenesis inhibitors were clearly defined. Recently, it has been reported that the antiangiogenic or antitumor effect of angiogenesis inhibitors may differ depending on the stages of tumor progression including angiogenic phenotype and tumor burden (14).

IFN-β has been used for patients with solid tumors including malignant gliomas with only marginal benefit, and a therapeutic guideline for the best clinical efficacy has not been determined (15, 16). There have been several in vitro (17, 18) and in vivo (19–24) studies demonstrating angiogenic activity of IFN-β in other tumors, and it has been reported that IFN-α and IFN-β can specifically down-regulate mRNA expression and protein production of bFGF in human bladder carcinomas.

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4 The abbreviations used are: bFGF, basic fibroblast growth factor; rhIFN-β, recombinant human interferon-β; VEGF, vascular endothelial growth factor; HUVECs, human umbilical vein endothelial cells.
(18, 24). However, the in vivo antiangiogenic property of IFN-β has not been studied in malignant gliomas. Here, we studied the in vivo antitumor effect and mechanism of rhIFN-β using orthotopic xenograft brain tumors generated by stereotactic intracerebral implantation of U-87 human glioma cells in nude mice.

MATERIALS AND METHODS

Brain Tumor Animal Model. A brain tumor animal model was made as described previously (11). Briefly, 6- to 8-week-old athymic nude (nu/nu) mice were housed in laminar-flow cabinets under specific-pathogen-free conditions. Animals were anesthetized by i.p. injection of xylazine (Rompun; Cutter Laboratories, Shawnee, KS) 12 mg/kg and ketamine (Ketalar; Parke-Davis & Co., Morris Plains, NJ) 30 mg/kg. Mice were held in a stereotactic frame with an ear bar. Human glioblastoma cell line U-87 (American Type Culture Collection, Manassas, VA), 2 × 10⁵ cells in a volume of 3 μl PBS, was injected slowly into the brain with a Hamilton syringe.

Treatment of Mice with rhIFN-β. To see whether the antitumor effect of IFN-β is influenced by the tumor burden or tumorigenesis stage, the animals were divided into two groups according to the time of treatment. Group 1 animals (n = 20) were treated from 7 days and those of group 2 (n = 20) from 21 days after the tumor cell inoculation. The mice of each group were treated with rhIFN-β (Beta-Feron; Cheiljeedang, Seoul, Korea) at 2 × 10⁶ IU (treated group, n = 10) or normal saline (control group, n = 10) i.p. once a day for 15 days. Two days after the final treatment, the animals were sacrificed, and the brains were removed. The presence of tumor in the brain was evaluated by serial coronal cutting with 1 mm thickness from the brain specimen. The midcoronal sections of the whole tumors were processed for histological and immunohistochemical analyses.

Tumor Size Measurement. The maximal area of the tumor in coronal section stained with H&E was selected and transcribed onto paper (×4.0 actual size) by a zoom stereo microscope (SZH-111; Olympus Optical Co., Tokyo, Japan) with drawing attachment (SZH-DA; Olympus Optical Co.). The transcribed area of the tumor was then measured with a planimeter (KP-21; Koizumi, Japan).

Immunohistochemical Staining. Formalin-fixed paraffin-embedded specimens obtained from the brains of the group 1 mice were studied as described previously (11). Immunohistochemical staining was performed by the avidin–biotin complex method for proliferation index, microvessel quantitation, and expression of VEGF and bFGF. The archived blocks were sectioned consecutively at a thickness of 5 μm and mounted on poly-l-lysine-coated slides. After routine deparaffinization, rehydration, and blockade of endogenous peroxidase activity, antigen retrieval was performed. Sections were subjected to microwave antigen retrieval by immersion in citrate buffer, pH 6.0. To detect Ki-67, VEGF, and bFGF proteins, we used 1:50 dilution of a rabbit polyclonal antibody for Ki-67 (Dako A/S, Denmark), VEGF (Santa Cruz Biotechnology, Santa Cruz, CA), and bFGF (Santa Cruz Biotechnology). For human von Willebrand factor (factor VIII), we used 1:300 dilution of the antibodies (Dako, Carpinteria, CA); Fast Red (Amresco, Solon, OH) was used as a chromogen. The slides were lightly counterstained with hematoxylin. All reagents except the primary antibody were used in the negative controls.

Ki-67 labeling index was determined by the percentage of immunopositive nuclei to the total number of nuclei in a high-power field (×400). An average of ~2000 nuclei were selected in each section randomly.

All blood vessels were determined by light microscopy according to the procedure described previously (25). After the area of highest neovascularization was identified, microvessels were counted at ×400 magnification. Any red-staining endothelial cell, or endothelial-cell cluster that was clearly separate from adjacent microvessels, tumor cells, and other connective tissue elements was considered a single, countable microvessel. Each count was expressed as the highest number of microvessels identified within a field. All counts were also performed by a pathologist who did not have any prior information about these specimens.

The immunohistochemical stained sections for VEGF and bFGF were rated on an arbitrary 0- to 3-point scale based on staining intensity that was interpreted as relative immunoreactivity by a pathologist blinded to the study. Ratings were designated as follows: no staining = 0, mild staining = 1, moderate staining = 2, and intense staining = 3.

Assessment of Apoptotic Cells. To determine the presence of apoptotic cells, we used the terminal deoxyribonucleotidyl transferase-mediated dUTP-biotin nick end labeling procedure, which allows in situ labeling of DNA breaks in tissue sections (26). We used the ApopTag in situ apoptosis detection kit (Oncor, Gaithersburg, MD), and all of the procedures were performed according to the manufacturer’s instructions. The slides were lightly counterstained with hematoxylin. In every specimen, ~5000 tumor cells were counted in at least 10 randomly chosen fields. The condensed nuclei were counted for this study because it was often difficult to compute the numbers of apoptotic cells derived from the fragmented nuclei.

Cell Proliferation Assay and Western Blotting. U-87, HUVECs, and PAM 212 murine keratinocytes (American Type Culture Collection) were seeded into six-well plates (1 × 10⁴ cells/ml) and allowed to adhere overnight. The cultures were then washed and refed with control medium or medium containing rhIFN-β at concentrations of 5–1000 IU/ml. Antiproliferative activity was determined by cell count assay from day 1 to day 10. U-87 cells (1 × 10⁵ cells/ml) were plated into 100-mm dishes and treated with medium containing rhIFN-β at concentrations of 0–500 IU/ml for 24 and 72 h; Western blot assay was performed as described previously (11). Immunodetection of VEGF and bFGF was accomplished with sc-152G goat polyclonal antibody for VEGF (1:1000 dilution in 1% BSA), sc-79G polyclonal antibody for bFGF (1:1000 dilution in 1% BSA; Santa Cruz), or mouse monoclonal antibody for bFGF (1:5000 dilution in 1% BSA; Sigma), followed by incubation with horseradish peroxidase-coupled rabbit anti-got IgG or rabbit antimouse IgG (Santa Cruz).

Statistical Analysis. The significance of the data was determined with Student’s t test (two-tailed) or the Wilcoxon rank sum test.
RESULTS

Effect of rhIFN-β on Brain Tumor Growth. Inhibitory effect of rhIFN-β on intracerebral glioma growth was significant in group 1, in which the treatment began 7 days after U-87 cell inoculation, and only minimal in group 2. The means of maximal area of tumors was 10.74 ± 2.88 mm² in control animals treated with saline and 3.27 ± 2.13 mm² in animals treated with rhIFN-β in group 1 (P = 0.029), whereas 16.43 ± 6.67 mm² in control animals and 14.65 ± 7.87 mm² in treated animals in group 2 (Fig. 1). Microscopically, the treated tumors showed minimally decreased cellularity, lymphocyte infiltration, and mitosis compared with controls. No necrosis was found in both treated and control tumors (data not shown).

Angiogenesis, Proliferation, and Apoptosis. We studied the effect of rhIFN-β on vascularity, proliferative activity, and apoptosis of the tumors in group 1. The number of blood vessels was 140.00 ± 14.27 in the tumors of control animals and 54.17 ± 17.23 in the treated tumors (P = 0.0001; Fig. 2A). The Ki-67 labeling index was 34.00 ± 5.33 in the control tumors and 36.33 ± 4.03 in the treated tumors (P = 0.4125; Fig. 2B). The apoptotic index was 1.28 ± 0.21% in the control tumors and 2.72 ± 0.27% in the treated tumors (P = 0.0001; Fig. 2C). To further determine the antiangiogenic property of rhIFN-β in mouse brain tumor models, we studied its effect on in vitro culture of glioma cells, human endothelial cells, and murine cells. As shown in Fig. 3, rhIFN-β inhibited the proliferation of U-87, HUVECs, and PAM 212 cells by >51.7%, 42.6%, and 46.8%, respectively, at doses of 100-1000 IU/ml.

Fig. 1 Growth inhibition of brain tumors by systemic administration of rhIFN-β. Seven days (group 1) and 21 days (group 2) after the stereotactic intracerebral injection of U-87 human glioma cells in nude mice, the animals received 2 × 10³ IU rhIFN-β or normal saline (control) i.p. once a day for 15 days. Two days after the last treatment, the animals were sacrificed and the brains were removed. In A, the tumor size was measured and calculated after H&E staining of a coronal section of the brain at the maximal brain tumor dimension (values shown are mean ± SD). B, representative brain tumors of control and treated animals stained with H&E. Arrowheads, the brain tumor. Bars, 1 mm; *, P = 0.029.
ysis. The point scale of each VEGF and bFGF expression of control tumors was $1.50 \pm 0.55$ and that of treated tumors was $1.33 \pm 0.52$ ($P = 0.6404$; Fig. 4A). The very small brain tumor tissues of the treated animals were not adequate for Western blot analysis, and instead we performed *in vitro* studies on U-87 cells. As shown in Fig. 4B, the level of VEGF and bFGF expression of U-87 cells was not influenced by rhIFN-β treatment at concentrations from 10–500 IU/ml.

**DISCUSSION**

IFNs are known to have multiple biological actions including modulation of gene expression and their protein products, immunomodulation, slowing of cell proliferation, and alterations in differentiation (27). The antiangiogenic property has been suggested to be another mechanism of the antitumor effect of IFNs. Several clinical observations have substantiated the antiangiogenic properties of IFNs. The chronic administration of IFN-α to patients with Kaposi sarcoma, pulmonary hemangiomatosis or life-threatening hemangiomas of infancy have resulted in regression of tumors (28–30). IFNs inhibit endothelial cell growth *in vitro* (17) and in experimental models of tumor-induced angiogenesis (19, 20). Treatment of tumor cells with IFNs *in vitro* before injection into untreated mice also inhibited tumor-induced angiogenesis, suggesting that IFNs can directly modulate the angiogenic potential of tumor cells (19). Both IFN-α and IFN-β have been known to possess common receptors (31) and tumoristatic activity against human glioma cells in culture (32) and xenograft in nude mice (33, 34). *In vitro* studies have suggested that IFN-β has a more potent growth-inhibitory effect than IFN-α when tested against human glioma cell lines (35, 36). IFN-β has been used in patients with malignant or recurrent glioma, but its therapeutic efficacy was only marginal (15, 16), and the molecular details associated with *in vivo* antglioma effects of IFN-β remain largely unknown.
Our results showed that suppression of brain tumor growth in nude mice by systemic treatment with rhIFN-β was more prominent in group 1, in which rhIFN-β treatment began 7 days post-tumor implant, but less significant in group 2, in which rhIFN-β treatment began 21 days post-tumor implant. In our preliminary experiments, the brain tumors of the control group were very small (1 mm^2) or invisible at 7 days postimplant and 10.74 ± 2.88 mm^2 at 21 days postimplant (data not shown). These results suggest that rhIFN-β may be tumoristatic rather than tumoricidal and may be more effective for the treatment of malignant glioma patients at an early stage with minimal or microscopic tumor burdens rather than at an advanced stage of tumor development. Our results also strongly support the notion of Bergers et al. (14) that antiangiogenic drugs may prove most efficacious when they are targeted to specific stages of cancers.

Despite the clinical activity of IFN-β on malignant gliomas (15, 16), its antitumor mechanism in vivo is still unclear. Böeethus et al. (37) reported that systemic administration of IFN-α to patients with glioblastoma multiforme induced marked changes in the tumor vasculature, which supports the notion that IFN-α may have an effect on tumor vessels. In our study, vascularity of the tumors was reduced to near one-third of the controls in vivo, and proliferation of HUVECs and PAM 212 murine cells was inhibited in vitro by rhIFN-β. Cook et al. (36) and we found that IFN-β inhibited proliferation of U-87 cells in vitro. Our results, showing that intracerebral tumor of nude mice maintained a high proliferation index after rhIFN-β therapy, is dissociated with the well-known in vitro antiproliferative effects of IFNs on tumor cells. Considering these in vitro and in vivo results, it can be postulated that the in vivo antiproliferative effect of rhIFN-β may differ from that of the in vitro, or the concentration of rhIFN-β administered may be lower than that necessary to inhibit tumor cell proliferation. Delivery of rhIFN-β to intracerebral tumor cells was reported to be hindered by the blood-brain barrier or blood-tumor barrier from the systemic circulation (38). These strongly suggest that the in vivo antitumor effect of rhIFN-β in malignant gliomas may be mediated, at least in part, via the angiogenesis inhibition rather than the antiproliferative activity on tumor cells and that rhIFN-β may inhibit angiogenesis more efficiently at early stage, before

Fig. 3 Antiproliferative effects of rhIFN-β. Human glioma cells (U-87 MG), HUVECs, and murine keratinocytes (PAM 212) of 1 × 10^6 cells/ml were seeded into six-well plates and allowed to adhere overnight. The cultures were then washed and refed with control medium or medium containing rhIFN-β at concentrations of 5–500 IU/ml from day 1 to day 10.

Fig. 4 rhIFN-β effect on bFGF and VEGF expressions in intracerebral tumors (in vivo) and U-87 cells (in vitro). In A, bFGF and VEGF expression was analyzed by immunohistochemical staining of the brain tumors of control and treated animals and assessed by the point scales based on staining intensity. In B, protein was extracted from the U-87 cell cultures after treatment with media containing rhIFN-β, 0–500 IU/ml, for 24 and 72 h, and Western blot analysis was done. The expression level of bFGF and VEGF protein was not affected by rhIFN-β treatment in malignant gliomas.
angiogenic phenotype change develops, than at advanced stage of tumor development.

IFNs have been known to inhibit angiogenesis not only by affecting endothelial cells but also by inhibiting production or release from tumor cells or lymphocytes of factors which may stimulate host response (19, 22). Slowing of the cell cycles and inhibition of cell proliferation have been known to be general properties of IFN activity (27, 39). However, the inhibition mechanism of endothelial cell growth by rhIFN-β has not been determined in detail. To determine whether rhIFN-β induces apoptosis in endothelial cells, we treated HUVECs with rhIFN-β and stained with 4′,6-diamidino-2-phenylindole (Sigma), in which apoptosis induction was not observed by the rhIFN-β treatment (data not shown). Contrary to the results of Singh et al. (18), which showed that IFN-α and IFN-β can specifically down-regulate mRNA expression and protein production of bFGF in bladder carcinoma, our study demonstrated that bFGF and VEGF expression of U-87 glioma was not influenced by rhIFN-β. These suggest that the mechanism of angiogenesis inhibition in vivo by rhIFN-β may be not simple and involve pathways other than bFGF and VEGF in human malignant gliomas.

Our results of a high cell proliferation index balanced by a high apoptosis rate in intracerebral tumors indicate that rhIFN-β may limit tumor growth by elevating the incidence of apoptosis to a steady state as a balance between apoptosis and proliferation of malignant glioma cells. The mechanism by which IFN therapy leads to an increase in tumor cell apoptosis is unknown, although it has been postulated that apoptosis is induced by IFN-γ in colorectal adenocarcinoma via the up-regulation of Fas and Bax antigen and down-regulation of Bcl-2 (40), and IFN-α and IFN-β can be expressed in the absence of antiproliferative effects on tumor cells in vivo and by our study demonstrating the antiangiogenic effect with apoptosis induction by rhIFN-β in intracerebral malignant gliomas. This pattern of tumor inhibition seems to be a general trait of various antiangiogenic therapies including TNP-470 (42), angiostatin (9, 11), and endostatin (10) which also result in increased apoptotic rates. The mechanism by which antiangiogenic therapy induces an increase in apoptosis in tumor cells is unknown. Induction of immunoglobulin kringles 1–3. Int. J. Cancer, 82: 538–543, 1993.

In conclusion, the in vivo antitumor effect of rhIFN-β in malignant glioma patients may be mediated via the inhibition of tumor angiogenesis, and its therapeutic efficacy may be strengthened by fine-tuning it to specific stages of the tumor progression. Further studies for detailed mechanisms underlying angiogenesis inhibition and apoptosis induction will be necessary to develop more efficient use of rhIFN-β for the treatment of malignant gliomas.

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