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siRNA Mediated Knockdown of Bcl-2 and Low-dose Taxol Treatment in Human Glioblastoma U251MG Cells Induces Apoptosis, Inhibits Cell Invasion, Angiogenesis, and Tumor Growth in Nude Mice

Joseph George, Naren L. Banik, Swapan K. Ray*

Department of Pathology, Microbiology and Immunology, University of South Carolina School of Medicine, Columbia, SC 29209, USA

*Department of Neurosciences, Medical University of South Carolina, Charleston, SC 29425, USA

Abstract

RNA interference using siRNA is a powerful tool to knockdown the mRNA and thus protein level of a target gene. Taxol is an anti-cancer drug that binds to β-tubulin to prevent tumor cell division; however, higher doses of taxol may be toxic to normal cells. The anti-apoptotic molecule Bcl-2 is upregulated in cancer cells for protection from apoptosis. The aim of our present study was to downregulate Bcl-2 expression using cognate siRNA in a highly invasive glioblastoma cell line (U251MG) during a low-dose taxol treatment and to examine apoptosis, inhibition of cell invasion, angiogenesis, and tumor growth. Human glioblastoma U251MG cells were treated with 100 nM taxol or 100 nM Bcl-2 siRNA or both for 72 h. Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) and Western blotting demonstrated around 80% knockdown of Bcl-2 mRNA and protein levels. Fluorescent activated cell sorting (FACS) analysis and the terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) assay demonstrated apoptosis in almost 60% of cells after combination treatment with taxol and Bcl-2 siRNA. Matrigel invasion studies demonstrated a significant decrease in cell invasion after treatment with taxol and Bcl-2 siRNA. In vivo angiogenesis assays in immunocompromised mice showed complete inhibition of neo-vasculature after treatment with both agents. The combination treatment with taxol and Bcl-2 siRNA further demonstrated a remarkable decrease in growth of both subcutaneous and intracerebral tumors in nude mice. Taken together, the results of our study indicated that the combination treatment with taxol and Bcl-2 siRNA effectively induced apoptosis and inhibited cell invasion, angiogenesis, and tumor growth. Therefore, this combination therapeutic strategy offers a potential tool for the controlling the growth of human glioblastoma. This work was supported by the R01 CA-91460 grant from the NCI.

Introduction

Malignant gliomas or glioblastomas are the most common primary brain tumors in adults and children and are associated with a dismal prognosis. Glioblastomas comprise 23% of primary brain tumors in the United States and are the most commonly diagnosed brain tumor in adults. Since tumor cells often infiltrate deep into the normal tissue, complete surgical removal of the brain tumor is almost impossible and responsible for the high incidence of recurrence.

Cell invasion, angiogenesis and tumor growth are complex mechanisms that involve a variety of biochemical and cellular processes. The degree of primary brain tumor growth is directly correlated with cell invasion and angiogenesis. Inhibition of these processes may not only suppress tumor growth but also improve the prognosis for advanced brain tumors. Thus, exploring methods to intervene in the process of cell invasion and angiogenesis would arrest the growth of glioblastomas.

Dysregulation of apoptotic mechanisms plays an important role in the pathogenesis and progression of various cancers as well as in the responses of tumors to therapeutic interventions. Highly invasive cancer cells are protected from apoptosis by upregulation of various anti-apoptotic molecules, such as B-cell lymphoma-2 (Bcl-2). Furthermore, Bcl-2 protects cells against taxol-mediated apoptosis by inducing multi-nucleation. Significant knockdown of the upregulated of Bcl-2 could pave an effective way to induce apoptosis in gliomas and thus to intervene in cell invasion, angiogenesis and tumor growth.

Taxol (paclitaxel) is a potent anticancer drug that strongly binds to the N-terminal region of β-subunit of tubulin and promotes the formation of highly stable microtubules that resist depolymerization, thus preventing active tumor cell division and arresting the cell cycle at the G2/M phase. The dynamic instability of microtubules affects the positioning of chromosomes during replication and inhibits cell division. Taxol also induces apoptosis in a wide spectrum of cancer cells by caspase-dependent and -independent apoptosis mechanisms. Although the progress of anticancer agents including taxol has improved therapeutic responses in advanced gliomas, the long-term prognosis remains unsatisfactory. Moreover, taxol also inhibits division of normal cells and causes undesirable side effects for patients. The use of a low dose taxol treatment during downregulation of Bcl-2 would be highly desirable to induce apoptosis and thus to prevent cell invasion, angiogenesis and tumor growth more effectively. The aim of our present investigation was to knockdown the upregulated Bcl-2 using gene specific siRNA during a low dose taxol treatment in U-251MG glioblastoma cells, to induce apoptosis, and to inhibit cell invasion, angiogenesis and tumor growth in immuno-compromised mice.

Figure 1

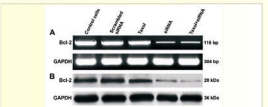

Figure 1. Expression of Bcl-2 mRNA and protein levels in U-251MG cells after treatment with100 nM taxol or transfection with a mammalian expression vector carrying Bcl-2 siRNA cDNA (pRNAT-CMV3.2/Neo, GenScript, Piscataway, NJ) or both together for 72 h. **(A) Semiquantitative RT-PCR.** Total RNA was isolated using BioRad Aurum kit. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression was used as an internal control. **(B) Western blotting for Bcl-2.** The blots were reprobed for GAPDH content to demonstrate equal loading of protein in all lanes.

Figure 1 demonstrates that transfection with Bcl-2 siRNA resulted in a 70% down regulation of Bcl-2 mRNA as well as protein levels. The treatment with both agents together resulted in 85% knockdown of Bcl-2 protein.

Figure 2

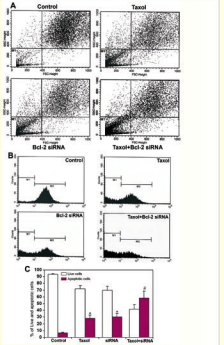

Figure 2 (A). **Flow cytometry dot plot of U-251MG cells after treatment with 100 nM taxol or transfection with a mammalian expression vector carrying Bcl-2 siRNA cDNA or both together for 72 h.** The cells were treated with 50 μg/ml propidium odide for 30 min at 4°C in dark. Column M1 represents the apoptotic cell population. A marked increase of cell population in the M1 area indicates increased apoptosis after treatment with taxol or transfection with Bcl-2 siRNA or both. The combined treatment of taxol and Bcl-2 siRNA resulted in more apoptotic cells than treatment with either alone. **(B) FACS histogram of U-251MG cells after treatment with taxol or transfection with Bcl-2 siRNA.** The prominent increase of sub-G1 phase population of cells (M1) indicates increased apoptosis after treatment with taxol or Bcl-2 siRNA or both. **(C) Quantitative representation of FACS data for live and apoptotic cells.** Data are representative of 4 independent experiments (*p<0.001 when compared to the control mean values and †p<0.001 when compared to taxol or Bcl-2 siRNA mean values).

Figure 3

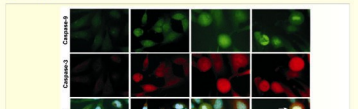

Figure 3. Double immunofluorescent staining to determine the occurrence of apoptosis in U-251MG cells. Double immunofluorescent staining to examine the expression of active subunits of caspase-9 and caspase-3. After treatments with taxol and Bcl-2 siRNA, the cells were fixed with 95% ethanol and blocked with 2% donkey and 2% goat serum in PBS (50:50) for 1 h. The cells were washed and treated with 1:50 diluted (1% bovine serum albumin in PBS) goat polyclonal cleaved caspase-9 (Santa Cruz Biotechnology) and 1:100 diluted rabbit monoclonal cleaved caspase-3 (Cell Signaling Technology) primary antibodies simultaneously and incubated overnight at 4C. The cells were then washed and incubated with PITC conjugated donkey anti-goat (MP Biomedicals, Solon, OH) and Texas red conjugated goat anti-rabbit (Biomeda, Foster City, CA) secondary antibodies at room temperature for 1 h to detect caspase-9 and caspase-3, respectively. Merged pictures demonstrated simultaneous expression of active subunits of caspase-9 and caspase-3 in apoptotic cells.

Figure 4

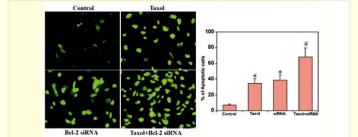

Figure 4. (A) Fluorescent TUNEL assay for detection of apoptotic cells after the treatments. The combined treatment with taxol and Bcl-2 siRNA resulted in more apoptotic cell death than either treatments alone. **(B)** Quantitation of TUNEL-positive cells using Image Pro-Discovery software. Data are representative of 4 independent experiments in duplicate (*p<0.001 when compared to the control mean values and †p<0.001 when compared to taxol or Bcl-2 siRNA mean values).

Figure 5

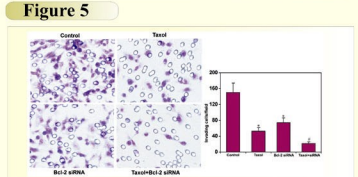

Figure 5. Tumor cell invasion assay. (A) Matrigel invasion assay was performed on U-251MG control cells or after treatment with 100 nM taxol or transfection with a mammalian expression vector carrying Bcl-2 siRNA cDNA or both agents together. Invasion assays were carried out in 12-well transwell inserts of polycarbonate filters with 12.0 i m pores coated with 200 μl of 0.1% matrigel. After a 48 h incubation period, the membranes were collected and stained with HEMA stain. The number of cells that migrated to the undersurface of the membrane were examined under a microscope, counted and photographed. A significant reduction in the number of invaded cells indicates the decreased invasive potency of U-251MG cells after treatment with taxol and Bcl-2 siRNA (B) Quantitative evaluation of matrigel invasion assay. The data represented are mean ± S.D. from 10 randomly selected microscopic fields from three independent wells (*p<0.001 when compared to the control

Figure 6

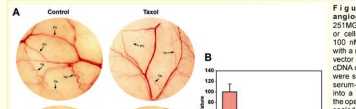

Figure 6. ***In vivo*** **angiogenesis assay.** U-251MG parental cells ($2x10^7$) or cells after treatment with 100 nM taxol or transfection with a mammalian expression vector carrying Bcl-2 siRNA cDNA or both agents together were suspended in 200 μL of serum-free medium, injected into a diffusion chamber and the opening was subsequently sealed with sterile bone wax. The diffusion chambers loaded with cells were surgically implanted under the dorsal skin of nude mice and left for 10 days. (A) Strong micro-vessel development (as indicated by arrows, TN) with curved thin structures arising from pre-existing vessels was observed in U-251MG parental cells. The formation of such microvasculature was considerably reduced and attenuated in both taxol and Bcl-2 siRNA treated cells and completely inhibited after treatment with both agents together. **(B) Quantitative representation of *in vivo* angiogenesis.** Tumor-induced neovasculature was measured in control, taxol and Bcl-2 siRNA treated cells. Values are mean S.D. of 6 animals from each group (p < 0.001). *TN: tumor-induced neovasculature; PV: pre-existing vasculature.*

Figure 7

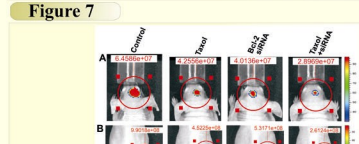

Figure 7. (A). Inhibition of intracerebral tumor in nude mice after combination treatment with taxol and Bcl-2 siRNA. U-251MG human glioblastoma cells were stably transfected with a mammalian expression vector (phCMV-FSR Vector, Genlantis, San Diego, CA) carrying luciferase gene and propagated in media containing G-418 at concentrations of 500 μg/ml. The stably transfected cells were treated with either 100 nM taxol or a mammalian expression vector carrying Bcl-2 siRNA cDNA (GenScript, Piscataway, NJ) or both agents together for 72 h. The cells were harvested and 1x10⁷ cells suspended in 100 l of serum free media were injected intracerebrally. Afterwards, the mice were injected intraperitoneally with either taxol (50 μg/injection/mouse) or Bcl-2 siRNA (50 μg DNA/injection/mouse) or both together for 20 days on alternate days. On day 21, the mice were injected with 100 μl of luciferin (Genlantis, San Diego, CA) at a concentration of 50mg/ml. After 10 min, the mice were visualized for luciferase activity using Xenogen IVIS-200 (Xenogen, Hopkinton, MA) imaging system. The combination treatment with taxol and Bcl-2 siRNA resulted in complete inhibition of intracerebral tumor formation in nude mice. The data are representative of 6 sets of animals in each group. **(B).** Inhibition of subcutaneous tumor in nude mice after combination treatment with taxol and Bcl-2 siRNA. The cells were cultured and treated as mentioned above. About 1x10⁷ cells suspended in 100 μl of serum free media were injected subcutaneously. Afterwards, the mice were injected intraperitoneally with either 100 nM taxol or Bcl-2 siRNA or both agents together for 20 days on alternate days. On day 21, the mice were injected with luciferin and visualized for tumor inhibition using Xenogen IVIS-200 imaging system. The individual treatments with both taxol and Bcl-2 siRNA resulted in a marked decrease of subcutaneous tumor growth, and the combination treatment with both agents resulted in complete inhibition of tumor formation in nude mice. The data are representative of 6 sets of experiments in each group.

Conclusions

- *Transfection of U-251MG cells with a mammalian expression vector carrying Bcl-2 siRNA cDNA resulted in about 70% downregulation of both Bcl-2 mRNA and protein levels.*
- *Double immunofluorescencent staining for active subunits of caspase-9 and caspase-3 demonstrated increased expression and co-localization of both molecules and induction of apoptosis after knockdown of Bcl-2.*
- *Combination treatment with taxol and Bcl-2 siRNA resulted in complete inhibition of both tumor cell invasion and formation of microvasculature.*
- *Simultaneous administration of low dose taxol and Bcl-2 siRNA significantly reduced subcutaneous and intracerebral tumor growth in nude mice.*
- *Combination of taxol and Bcl-2 siRNA offers a novel therapeutic tool for controlling growth of glioblastomas.*

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