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Synergistic Effect of Bcl-xL small interfering RNA and Genistein in Human Neuroblastoma SH-SY5Y and SK-N-DZ Cells for Induction of Apoptosis and Inhibition of Angiogenesis and Tumor Growth in Nude Mice

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Abstract

Genistein is a phytoestrogenic isoflavone with anti-cancer properties. The anti-apoptotic molecule Bcl-xL is upregulated in many cancers including neuroblastoma to provide protection from apoptosis. The aim of our present study was to downregulate Bcl-xL using cognate siRNA during genistein treatment in two highly invasive neuroblastoma cell lines SH-SY5Y and SK-N-DZ, and to examine apoptosis, and inhibition of angiogenesis, and tumor growth in nude mice. The cells in cultures were treated with 100 nM Bcl-xL siRNA or 100 µM genistein or both agents together for 48 h. Morphological analysis and TUNEL assay demonstrated apoptosis in about 60% of cells after combination treatment with Bcl-xL siRNA and genistein. Apoptosis was associated with increase in Bax:Bcl-2 ratio, mitochondrial release of cytochrome c, and activation of caspase-9 and caspase-3 through the intrinsic pathway. Genistein also triggered the extrinsic pathway leading to apoptosis through upregulation of FasL and TNF-α associated death domains and activation of caspase-8. Furthermore, treatment with Bcl-xL siRNA and genistein resulted in significant increases of tBid, cleaved lamin, caspase-3-activated DNase (CAD) and cleaved poly (ADP-ribose) polymerase (PARP) indicating occurrence of apoptotic cell death through activation of both extrinsic and intrinsic pathways. In vivo angiogenesis in nude mice demonstrated formation of neo-vasculature with untreated neuroblastoma cells and complete inhibition after treatment with Bcl-xL siRNA and genistein. Administration of Bcl-xL siRNA and genistein also showed a remarkable decrease of subcutaneous tumor growth in immunocompromised mice. Thus, our study demonstrated that combination treatment with Bcl-xL siRNA and genistein worked synergistically to induce apoptosis and inhibit angiogenesis, and decreases tumor growth and therefore could serve as potential therapeutic tool for controlling the growth of human neuroblastomas. This work was supported by the R01 NS-57811 grant from the NINDS.

Introduction

Neuroblastomas are the most common extracranial solid, malignant tumors that mainly affect children and are the most common cancers in infancy with a dismal prognosis. Neuroblastoma is often present at birth, but is most often diagnosed much later when the child begins to show symptoms of the disease. About 50 percent of neuroblastoma cases occur in children younger than two years old. Neuroblastomas are neuroendocrine tumor, arising from any neural crest element of the sympathetic nervous system (SNS). In most cases, neuroblastomas have already metastasized outside of the original site at the time of diagnosis.

Dysregulation of apoptotic mechanisms plays a significant role in the progression neuroblastomas as well as in the responses of these solid tumors to therapeutic interventions. Highly invasive tumor cells are protected from apoptosis by upregulation of various anti-apoptotic molecules, such as B-cell lymphoma-extra large (Bcl-xL) protein. Significant knockdown of the over expression of Bcl-xL could pave an effective way to induce apoptosis in neuroblastomas and thus make the tumor cells more prone to therapeutic agents.

Genistein is a phytoestrogenic isoflavone with reported anti-cancer properties. The aim of our present investigation was to knockdown the over expression of Bcl-xL molecule using a mammalian expression vector carrying Bcl-xL siRNA cDNA in combination with genistein treatment in human malignant SH-SY5Y and SK-N-DZ cells to induce apoptosis and inhibit angiogenesis and tumor growth in immuno-compromised mice.

Figure 1

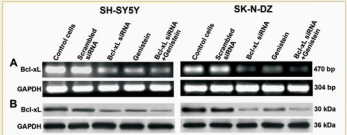

Figure 1. Expression of Bcl-xL mRNA and protein levels in SH-SY5Y and SK-N-DZ cells after transfection with a mammalian expression vector carrying Bcl-xL siRNA cDNA (pRNAT-CMV3.2/Neo, GenScript, Piscataway, NJ) or treatment with 100 µM genistein (Sigma, St. Louis, MO) or both agents together for 48 h. **(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-xL.** The blots were reprobed for GAPDH content to demonstrate equal loading of protein in all lanes.

Figure 1 demonstrates that transfection with Bcl-xL siRNA resulted in a 75% down regulation of Bcl-xL mRNA as well as protein levels. The treatment with both agents together resulted in 85% knockdown of Bcl-xL mRNA and protein levels in both cell lines.

Figure 2

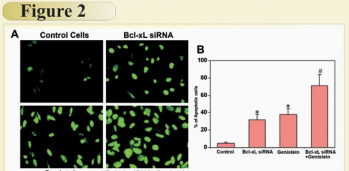

Figure 2. (A) Terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) assay for detection of apoptotic cells after transfection with a mammalian expression vector carrying Bcl-xL siRNA or treatment with 100 µM genistein or both together for 48 h in SH-SY5Y cells. The combination treatment with Bcl-xL siRNA and genistein resulted in marked increase in apoptotic cell death than either treatment 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 Bcl-xL siRNA or genistein mean values).

Figure 3

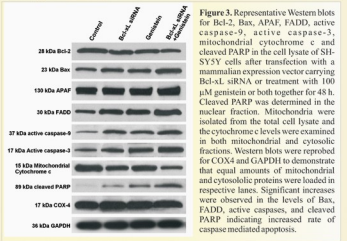

Figure 3. Representative Western blots for Bcl-2, Bax, APAF, FADD, active caspase-9, active caspase-3, mitochondrial cytochrome c and cleaved PARP in the cell lysate of SH-SY5Y cells after transfection with a mammalian expression vector carrying Bcl-xL siRNA or treatment with 100 µM genistein or both together for 48 h. Cleaved PARP was determined in the nuclear fraction. Mitochondria were isolated from the total cell lysate and the cytochrome c levels were examined in both mitochondrial and cytosolic fractions. Western blots were reprobed for COX4 and GAPDH to demonstrate that equal amounts of mitochondrial and cytosolic proteins were loaded in respective lanes. Significant increases were observed in the levels of Bax, FADD, active caspases, and cleaved PARP indicating increased rate of caspase mediated apoptosis.

Figure 4

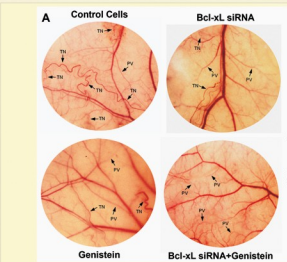

Figure 4. (A) In vivo angiogenesis assay. SH-SY5Y parental cells ($2x10^6$) or cells after transfection with a mammalian expression vector carrying Bcl-xL siRNA or treatment with 100 µM genistein or both agents together were suspended in 200 µL of serum-free medium, injected into a diffusion chamber 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. Strong micro-vessel development (as indicated by arrows, TN) with curved thin structures arising from pre-existing vessels was observed in SH-SY5Y parental cells. The formation of such microvasculature was considerably reduced and attenuated in both Bcl-xL siRNA and genistein 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, Bcl-xL siRNA and genistein treated cells (*p<0.001 when compared to the control mean values). *TN: tumor-induced neovasculature; PV: pre-existing vasculature.*

Figure 5

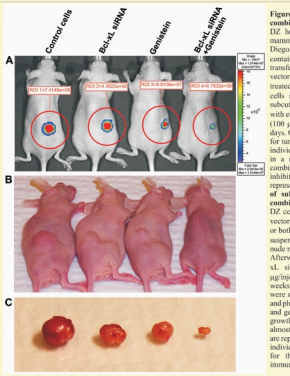

Figure 5. (A) Inhibition of subcutaneous tumor in nude mice after combination treatment with Bcl-xL siRNA and genistein. SK-N-DZ human neuroblastoma cells were stably transfected with a mammalian expression vector (pbCMV4/FSR Vector, GenInntis, San Diego, CA) carrying luciferase gene and propagated in media containing G-418 at concentrations of 500 µg/ml. The stably transfected cells were again transfected with a mammalian expression vector carrying Bcl-xL siRNA cDNA (GenScript, Piscataway, NJ) or treated with 100 µM genistein or both together for 48 h. About $1x10^7$ cells suspended in 100 µL of serum free media were injected subcuteanously. Afterwards, the mice were injected intraperitoneally with either Bcl-xL siRNA (50 µg DNA/injection/mouse) or genistein (100 µg/injection/mouse) or both 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 Bcl-xL siRNA and genistein resulted in a marked decrease of subcutaneous tumor growth, and the combination treatment with both agents together resulted in complete inhibition of tumor formation in nude mice. The data are representative of 4 sets of experiments in each group. **(B) Inhibition of subcutaneous solid tumor formation in nude mice after combination treatment with Bcl-xL siRNA and genistein.** SK-N-DZ cells in culture were transfected with a mammalian expression vector carrying Bcl-xL siRNA cDNA or treated with 100 µM genistein or both agents together for 48 h. About $1x10^7$ control or treated cells suspended in 100 µL of matrigel were injected subcutaneously into nude mice. The animals were left for 3 weeks without any treatment. Afterwards, the mice were injected intraperitonally with either Bcl-xL siRNA (50 µg DNA/injection/mouse) or genistein (100 µg/injection/mouse) or both agents together on alternate days for 5 weeks. The animals were sacrificed at the end of 8th week, tumors were surgically removed, tumor weight and volume were measured and photographed. The individual treatments with both Bcl-xL siRNA and genistein resulted in a marked decrease of subcutaneous tumor growth, and the combination treatment with both agents resulted in almost complete inhibition of tumor formation in nude mice. The data are representative of 4 sets of experiments in each group. (B) Effect of individual and combination treatments of Bcl-xL siRNA and genistein for the suppression of subcutaneous tumor development in immunosuppressed mice.

Figure 6

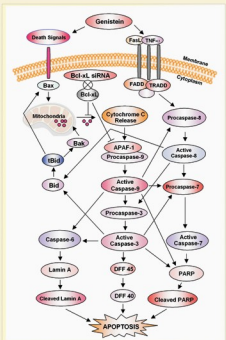

Figure 6. Molecular mechanism of Bcl-xL siRNA and genistein in the induction of caspase mediated apoptosis. Genistein upregulates FasL and TNF-α, which modifies the death receptor/death domain activities of the cell surface molecules, FADD and TRADD resulting in the activation of extrinsic caspase pathway. Bcl-xL siRNA downregulates the anti-apoptotic molecule Bcl-xL and accelerates the release of cytochrome c from mitochondria. The association of cytosolic cytochrome c with procaspase-9 and APAF-1 processes procaspase-9 to its active form, which then initiates the intrinsic pathway of caspase-mediated apoptosis. Procaspase-3 is cleaved to its active form by elevated caspase-9 as well as caspase-8. The active caspase-3 in turn cleaves DFF45 and PARP releasing the active subunits DFF40 and PARP, which trigger DNA fragmentation and apoptosis.

Conclusions

- *Combination treatment with Bcl-xL siRNA and Genistein resulted in about 85% knockdown of Bcl-xL mRNA and protein levels in both SH-SY5Y and SK-N-DZ cells.*
- *Combination treatment with Bcl-xL siRNA and Genistein resulted in apoptosis of 70% cells.*
- *Combination treatment with Bcl-xL siRNA and Genistein resulted in complete inhibition of tumor-induced neovasculature.*
- *Simultaneous administration of Bcl-xL siRNA and Genistein significantly reduced subcutaneous tumor growth in nude mice.*
- *Combination of Bcl-xL siRNA and Genistein offers a novel therapeutic tool for treatment of neuroblastomas.*

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