AACR 2009 # 4662

Survivin knockdown enhanced efficacy of N-(4-hydroxyphenyl) retinamide for apoptosis in human glioblastoma U118MG and U251MG cells and inhibited invasion, angiogenesis, and tumor growth

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Abstract

Upregulation of the anti-apoptotic molecule survivin inhibits apoptosis and promotes tumor growth. Survivin is selectively upregulated in various cancers including glioblastoma, which is the most malignant brain tumor characterized by high angiogenesis, invasion, and cell proliferation. Treatment of cancer cells with N-(4-hydroxyphenyl) retinamide (4-HPR) induces apoptosis due to destabilization of mitochondrial membrane and activation of caspase mediated apoptotic pathways. In the present investigation, we examined whether survivin knockdown using cognate siRNA could enhance efficacy of 4-HPR for apoptosis in human glioblastoma U118MG and U251MG cells and inhibit cell invasion, angiogenesis, and tumor growth in athymic nude mice. Using a plasmid vector expressing cognate siRNA, we down regulated expression of survivin in both U118MG and U251MG cells and simultaneously treated them with 1 μM 4-HPR for 48 h. Following the treatment of cells, we performed MTT assay, double immunofluorescent staining, TUNEL staining, FACS analysis, and matrigel invasion assay. We also carried out Western blotting for examining the molecules involved in caspase mediated apoptotic pathways. Double immunofluorescent stainings showed marked decrease in survivin expression and conspicuous increase in active caspase-3 after transfection of cells with survivin siRNA and treatment with 4-HPR. Both TUNEL staining and FACS analysis demonstrated apoptosis in more than 80% of cells after treatment with combination of both agents. MTT assay and invasion studies demonstrated marked decreases in tumor cell proliferation and invasion, respectively. Thereafter, we performed in vivo angiogenesis and tumor regression studies in athymic nude mice. Angiogenesis studies under the dorsal skin of athymic nude mice depicted remarkable inhibition of neovascularization after treatment with combination of survivin siRNA and 4-HPR. In vivo imaging studies on intracerebral and subcutaneous tumorigenesis and also longitudinal studies on solid tumor development showed marked decreases in tumorigenesis and solid tumor development after treatment with combination of survivin siRNA and 4-HPR. Therefore, simultaneous survivin knockdown using cognate siRNA and 4-HPR treatment could be a novel therapeutic strategy for controlling growth of human glioblastomas. This investigation was supported in part by the R01 grants (CA-91460 and NS-57811) from the National Institutes of Health (Bethesda, MD).

Introduction

Glioblastomas are very heterogenous and the most common form of malignant primary brain tumors. A major challenge in patients with glioblastomas is the propensity of the tumor cells to invade rapidly deep into the surrounding tissues. Invasive tumor cells escape surgical removal and, because of their increased resistance to apoptosis, they are relatively resistant to radiation and chemotherapy. It is necessary to develop explicit treatment methods targeting the specific molecular aberrations that underlie the pathogenesis of glioblastoma. Development of appropriate combination therapeutic strategies including gene therapy would definitely help to decrease the aggressive behavior of this dreaded tumor, thereby identify an effective treatment.

Survivin is a member of the family of inhibitor of apoptosis (IAP) proteins and it functions as a key regulator of mitosis and programmed cell death. The role of survivin in cancer pathogenesis is not limited to apoptosis inhibition but also involves the regulation of the mitotic spindle checkpoint and the promotion of angiogenesis and chemoresistance. Survivin is highly expressed in most human tumors and fetal tissue, but is completely absent in terminally differentiated cells. Tumors that highly express survivin generally bear a poor prognosis and are associated with resistance to radiation and chemotherapy. Survivin gene expression is transcriptionally repressed by wild-type p53 and can be deregulated in cancer by several mechanisms, including gene amplification, hypomethylation, increased promoter activity, and loss of p53 function. Therefore, survivin makes an ideal target for cancer therapy targeting tumor cells alone while leaving normal cells unaffected.

The synthetic retinoid, N-(4-hydroxyphenyl) retinamide (4-HPR) has high retinoid activity with low pharmacological toxicity and is a powerful agent to induce tumor cell apoptosis in various cancers. Many animal and clinical studies have shown that 4-HPR directly interacts with cell proliferation and growth, inhibiting angiogenesis and malignant tumor growth. Treatment of tumor cells with 4-HPR results in induction of apoptosis through destabilization of mitochondrial membrane, release of mitochondrial cytochrome c and activation of intrinsic caspase mediated apoptotic pathway leading to apoptosis. However, treatment with 4-HPR may induce apoptosis in normal cells also. This could be solved by using a low dose of 4-HPR and simultaneously adopting a mechanism to induce apoptosis exclusively targeting tumor cells.

Small interfering RNA (siRNA) are synthetic antisense oligonucleotides that silence the expression of a particular gene by its complementary binding and cleavage, which results in disruption of translation of the targeted gene. The purpose of this investigation was to induce apoptosis through knockdown of survivin using cognate siRNA and simultaneous use of 4-HPR in two highly invasive human glioblastoma cell lines, U251MG and U118MG and to examine whether such a combination could inhibit cell invasion, angiogenesis and tumor growth in immunosuppressed mice. Our study was also aimed to elucidate the mechanism of inhibition of angiogenesis and tumor growth in vivo after the combination treatment with survivin siRNA and 4-HPR.

Figure 1

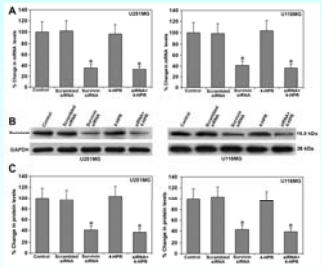

Figure 1. Levels of survivin mRNA and protein levels in U251MG and U118MG cells after transfection with a plasmid vector carrying survivin siRNA cDNA or treatment with 1 μM 4-HPR or both agents together for 48 h. **(A)** Real-time RT-PCR analysis for survivin mRNA. Values are mean ± S.D. of 6 assays in each group (*p<0.001 compared to the control mean values). **(B)** Western blotting for survivin. The blots were reprobed for GAPDH content to demonstrate that all samples were loaded with equal amount of protein. The data are representative of 6 independent experiments. **(C)** Quantitative evaluation of Western blotting. Values are mean ± S.D. of 6 assays in each group (*p<0.001 compared to the control mean values).

Figure 2

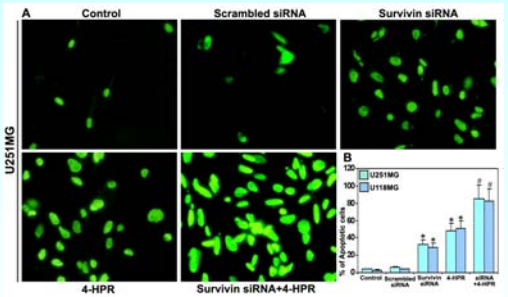

Figure 2. **(A)** TUNEL staining for detection of apoptotic cells after transfection with survivin siRNA or treatment with 1 μM 4-HPR or both together. TUNEL staining was performed using a fluorometric TUNEL kit. The combination treatment with survivin siRNA and 4-HPR resulted in more apoptotic cell death than either treatment alone. **(B)** Quantitation of TUNEL-positive cells using Image-Pro Discovery software. Data are mean ± S.D. of 6 independent experiments in each group (*p<0.001 compared to the control mean values and #p<0.001 compared to survivin siRNA or 4-HPR mean values).

Figure 3

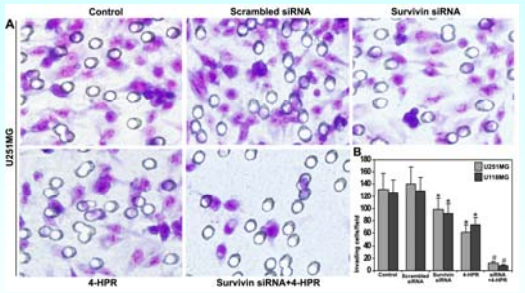

Figure 3. **(A)** Tumor cell invasion assay for U251MG and U118MG cells transfected with a plasmid vector expressing surviving siRNA or treated with 1 μM 4-HPR or both agents together. Invasion assays were carried out in 12-well transwell inserts of polycarbonate filters with 12.0 μm pores coated with 200 μl of 0.1% matrigel. After a 48 h incubation period, the transwell membranes were collected and stained. A significant reduction in the number of invaded cells indicates the decreased invasive potency of the treated cells. **(B)** Quantitative evaluation of matrigel invasion assay. The data are mean ± S.D. of 10 randomly selected microscopic fields from three independent wells in each group (*p<0.001 compared to the control mean values and #p<0.001 compared to survivin siRNA or 4-HPR mean values).

Figure 4

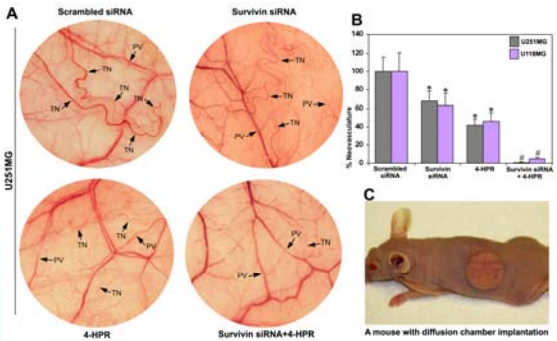

Figure 4. **(A)** In vivo angiogenesis assay. U251MG cells transfected with a plasmid vector expressing survivin siRNA or treated with 4-HPR or both agents together were suspended in 200 μl of serum-free medium, injected into a diffusion chamber, implanted under the dorsal skin of nude mice and left for 10 days. Strong development of new microvessels (arrows, TN) arising from pre-existing vessels as curved thin structures in zigzag pattern was observed in U251MG control and scrambled siRNA transfected cells. The formation of such microvasculature was considerably reduced in both survivin siRNA and 4-HPR treated cells and almost completely inhibited after combination treatment with both agents. **(B)** Quantitative representation of in vivo angiogenesis. Tumor-induced neovasculature was measured with the help of an ocular micrometer. Values are mean ± S.D. of 6 samples from each group (*p<0.001 compared to the control mean values and #p<0.001 compared to survivin siRNA or 4-HPR mean values). *TN: tumor-induced neovasculature; PV: pre-existing vasculature.* **(C)** An immunocompromised mouse bearing a diffusion chamber implanted under the dorsal skin for the assay of in-vivo angiogenesis.

Figure 5

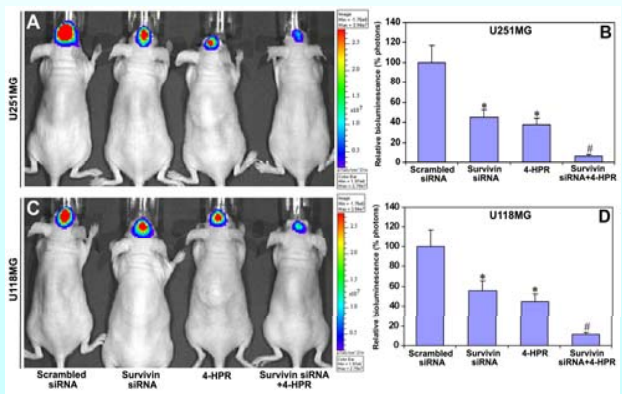

Figure 5. Inhibition of intracerebral tumorigenesis in athymic nude mice after the treatment with survivin siRNA and/or 4-HPR. U251MG or U118MG cells stably transfected with luciferase gene were injected into the intracerebrum of nude mice. Beginning from day 3, the mice were injected either with survivin siRNA plasmid vector (5 μg DNA/injection/mouse) or 4-HPR (1 μg/injection/mouse) or both agents together for 20 days on alternate days at the site of tumor cell implantation. Then the mice were injected intraperitoneally with 100 μl (50 mg/ml) of luciferin and visualized for the effect of treatments using Xenogen IVIS–200 imaging system. The data are representative of 6 sets of mice in each group. The background bioluminescence signal from an untreated mouse is about 1.5e+05 photons on Xenogen IVIS-200 imaging machine. **(A)** & **(C)** Animals transplanted with U251MG cells and U118MG cells, respectively. **(B)** & **(D)** Quantitative representation of relative bioluminescence as percentage photons in both U251MG and U118MG cells, respectively, after the treatment with survivin siRNA and/or 4-HPR. Data are mean ± S.D. of 6 animals in each group (*P < 0.001 compared to the scrambled siRNA mean values and #P < 0.001 compared to survivin siRNA or 4-HPR mean values).

Figure 6

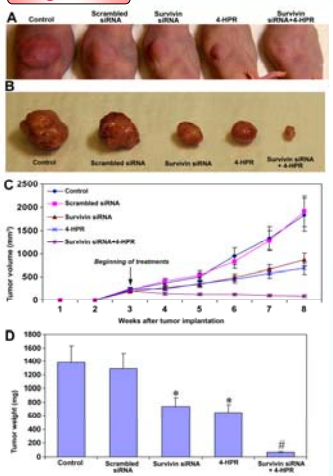

Figure 6. **(A)** Inhibition of subcutaneous solid tumor development in nude mice after the treatment with survivin siRNA and/or 4-HPR. U118MG cells were harvested, counted, suspended in an equal volume of high concentrated Matrigel and 100 μl of the suspension (5x106 cells) was injected under the dorsal skin of nude mice. The animals were left for 3 weeks without any treatment for uniform development of visible tumors. Afterwards, the mice were injected at the tumor site with either survivin siRNA plasmid vector (5 μg DNA /injection/mouse) or 4-HPR (1 μg /injection/mouse) or both agents together on alternate days for 5 weeks. At the end of 8th week, the animals were anesthetized with ketamine and xylazine and then photographed. **(B)** The tumors were surgically removed, weighed, and photographed. The data are representative of 6 sets of mice in each group. **(C)** Longitudinal measurement of tumor volume using a digital vernier caliper in nude mice after the combination treatment with survivin siRNA and/or 4-HPR. Data are mean ± S.D. of 6 animals in each group. **(D)** Quantitative representation of tumor weight. Data are mean ± S.D. of 6 animals in each group (*P<0.001 compared to the control mean values and #P<0.001 compared to survivin siRNA or 4-HPR mean values).

Conclusions

- *Knockdown of survivin using cognate siRNA resulted in about 60% decrease of survivin mRNA and protein levels in both U251MG and U118MG cells (Fig. 1)*
- *Combination treatment with survivin siRNA and 4-HPR resulted apoptosis in 80% tumor cells (Fig. 2).*
- *Combination treatment with survivin siRNA and 4-HPR resulted marked decrease in tumor cell invasion on matrigel (Fig. 3)*
- *Combination treatment with survivin siRNA and 4-HPR resulted in near complete inhibition of tumor-induced neovasculature (Fig. 4).*
- *Simultaneous administration of survivin siRNA and 4-HPR significantly decreased orthotopic tumorigenesis in nude mice (Fig. 5).*
- *Combination treatment with survivin siRNA and 4-HPR resulted in marked decrease of solid tumor growth in the subcutaneous area of nude mice (Fig. 6).*

Presented at the 100th Annual meeting of the American Association for Cancer Research held at Denver, CO, April 18-22, 2009