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Combination of 4-HPR and Genistein Increases Apoptosis in Glioblastoma Cells and Inhibits Invasion, Angiogenesis, and Tumor Growth

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Abstract

N-(4-Hydroxyphenyl) retinamide (4-HPR) is a synthetic retinoid that induces apoptosis in cancer cells. Genistein (GST) is an isoflavone that has anti-cancer effects. We examined whether combination of 4-HPR and GST could increase apoptosis in human glioblastoma A172 and U87MG cells and also inhibit cell invasion, angiogenesis, and tumor growth. After treatment of cells with 4-HPR, GST, or combination of both, we performed MTT assay, TUNEL staining, FACS analysis, Western blotting for apoptosis related proteins, and matrigel invasion assay. MTT assay showed significant decrease in cell viability due to treatment with combination of 4-HPR and GST. TUNEL and FACS analysis demonstrated apoptosis in more than 80% cells after treatment with combination of drugs. Apoptosis was associated with increases in Bax:Bcl-2 ratio, mitochondrial release of cytochrome c, and activation of caspase-9 and caspase-3. Matrigel invasion assay showed decrease in cell invasion after treatment with the combination. In vivo angiogenesis assay indicated inhibition of neovascularization in glioblastoma xenografts in nude mice after treatment with both agents. Intracerebral and subcutaneous tumorigenesis and also solid tumor development in nude mice were decreased due to treatment with combination of drugs. Thus, combination of 4-HPR and GST induced caspase-mediated apoptosis in glioblastoma cells and inhibited cell invasion, neovascularization, and tumor growth. So, this combination can be used as a potential therapy for effective treatment of glioblastomas. This study was supported by the NIH grants (CA-91460 and NS-57811).

Introduction

Human glioblastoma is a very heterogenous and the most common primary brain tumor in adults with a dismal prognosis. Glioblastomas are very aggressive tumors tend to invade rapidly deep into the surrounding tissues, making them deadly and severely impairing the quality of life of the affected individual. Current treatment modalities including surgery, radiation and chemotherapy, offer only palliation. It is necessary to develop explicit treatment methods targeting the specific molecular aberrations that underlie the pathogenesis of glioblastoma. Development of appropriate combination therapy and gene therapy would definitely help to decrease the aggressive behavior of this dreaded tumor and probably an effective treatment.

The synthetic retinoid, N-(4-hydroxyphenyl) retinamide (4-HPR), has high retinoid activity with low pharmacological toxicity and is a promising agent for cancer prevention and treatment. Many animal and clinical studies have shown that 4-HPR directly interacts with cell proliferation and growth, inhibiting 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. So combination of 4-HPR with another effective cancer preventive agent with a distinct molecular mechanisms would be an appropriate strategy to prevent and treat various cancers.

Genistein (4',5,7-trihydroxyisoflavone), an isoflavone abundant in soy products, has anti-tumor properties in several types of cancers. Genistein is a specific inhibitor of tyrosine kinases, enzymes involved in the phosphorylation of intracellular proteins that triggers a cascade of events leading to changes in gene expression. It has been shown that dietary concentrations of genistein can inhibit prostate cancer metastasis. Furthermore, genistein inhibits invasive potential of human hepatocellular carcinoma by altering cell cycle, apoptosis, and angiogenesis. However the effect of genistein on glioblastoma has not been studied.

Our previous studies proved that combination therapy is more effective for the induction of apoptosis and suppression of angiogenesis and tumor growth in glioblastoma than single drug treatments. So, we anticipated that combination treatment with two new powerful anticancer agents, 4-HPR and genistein, would be very effective to induce apoptosis and inhibit angiogenesis and tumor growth in glioblastoma. The aim of our present investigation was to trigger both intrinsic and extrinsic caspase mediated pathway leading to apoptosis using a combination treatment with 4-HPR and genistein in two malignant glioblastoma cells, A172 and U87MG and to examine the inhibition of angiogenesis and tumor growth in immunosuppressed mice.

Figure 1

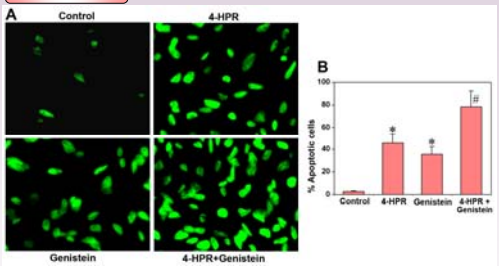

Figure 1. **(A)** TUNEL staining for detection of apoptotic cells after treatment with 1 μM 4-HPR or 10 μM genistein or both agents together. **(B)** Quantitation of TUNEL-positive cells using Image-Pro Discovery software. Data are mean ± S.D. of 6 independent experiments in duplicate (*$P < 0.001$ when compared to the control mean values and # $P < 0.001$ compared to 4-HPR or genistein mean values).

Figure 2

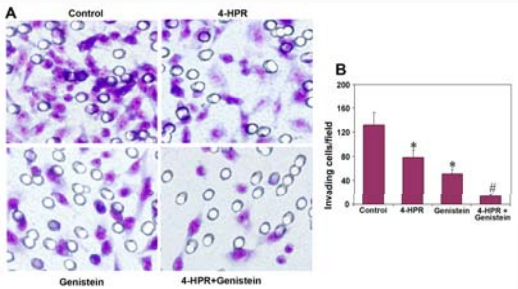

Figure 2. **(A)** Tumor cell invasion assay for A172 cells treated with 1 μM 4-HPR or 10 μM genistein or both together. Invasion assays were carried out in 12-well transwell inserts of polycarbonate filters with 12.0 μm pores coated with 200 μl of 0.1% matrigel. After a 48 h incubation period, the membranes were collected and stained. A significant reduction in the number of invaded cells indicates the decreased invasive potency. **(B)** Quantitative evaluation of matrigel invasion assay. The data represented are mean ± S.D. from 10 randomly selected microscopic fields from three independent wells (*$P < 0.001$ compared to the control mean values and #$P < 0.001$ compared to 4-HPR or genistein mean values).

Figure 3

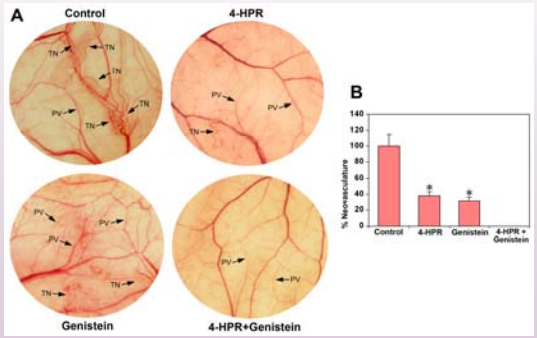

Figure 3. (A) Inhibition of in vivo angiogenesis in the dorsal skin of nude mice. Strong micro-vessel development (as indicated by arrows, TN) with curved thin structures arising from pre-existing vessels (PV) was observed in A172 untreated cells. The development of such neovasculature was significantly reduced in both 4-HPR or genistein treated cells and completely inhibited after combination treatment of both agents. (B) Quantitation 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). *TN: tumor-induced neovasculature; PV: pre-existing vasculature.*

Figure 4

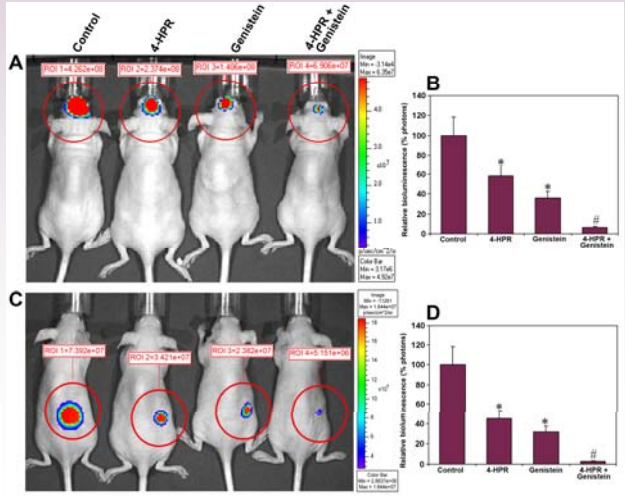

Figure 4. Inhibition of intracerebral **(A)** and subcutaneous **(C)** tumorigenesis in immunosuppressed mice after treatment with 4-HPR and/or genistein. U87MG cells were stably transfected with luciferase gene and injected into the intracerebrum or in the subcutaneous area of mice. Beginning from day 3, the mice were injected either with 4-HPR (1 μg/injection/mouse) or genistein (10 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 with luciferin and visualized for the effect of treatments using Xenogen IVIS–200 imaging system. The data are representative of 6 sets of animals in each group. **(B & D)** Quantitative representation of relative bioluminescence as percentage photons after treatment with 4-HPR and/or genistein in intracerebral and subcutaneous tumorigenesis, respectively. 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 with 4-HPR or genistein mean values).

Figure 5

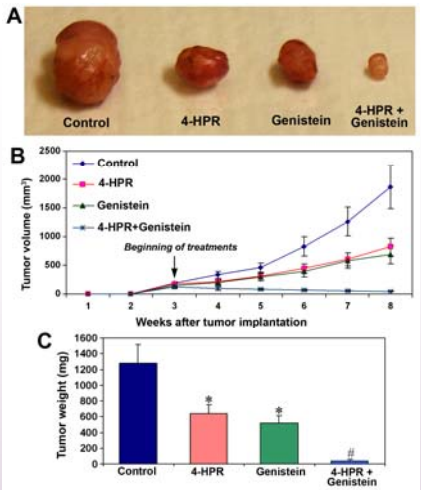

Figure 5. **(A)** Inhibition of subcutaneous solid tumor development in nude mice after the treatments with 4-HPR and/or genistein. U87MG cells were harvested, counted, suspended in an equal volume of high concentrated Matrigel and 100 μl of this suspension (5x106 cells) was injected under the dorsal 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 either with 4-HPR (1 μg/injection/mouse) or genistein (10 μg/injection /mouse) or both agents together on alternate days for 5 weeks. At the end of the 8th week, the tumors were surgically removed, weighed, and photographed. The data are representative of 6 animals in each group. **(B)** Longitudinal measurement of tumor volume using a digital vernier caliper in nude mice after treatments with 4-HPR and/or genistein. Data are mean ± S.D. of 6 animals in each group. **(C)** Quantitative representation of tumor weight. Data are Mean ± S.D. of 6 animals in each group (*$P < 0.001$ when compared to the control values and #$P < 0.001$ compared with 4-HPR or genistein mean values).

Conclusions

- *Combination treatment with 4-HPR and genistein resulted in apoptosis of 80% tumor cells.*
- *Combination treatment with 4-HPR and genistein resulted in marked decrease of tumor cell invasion.*
- *Combination treatment with 4-HPR and genistein resulted in complete inhibition of tumor-induced neovasculature.*
- *Simultaneous administration of 4-HPR and genistein resulted in significant reduction of intracerebral and subcutaneous tumorigenesis in nude mice.*
- *Combination treatment with 4-HPR and genistein resulted in marked decrease of solid tumor development in the subcutaneous area of nude mice.*

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