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Combination of 4-HPR and EGCG induces apoptosis and inhibits angiogenesis and tumorigenesis of neuroblastoma SH-SY5Y and SK-N-DZ cells

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Abstract

Neuroblastomas are extracranial, malignant solid tumors in childhood with a dismal prognosis. N-(4-Hydroxyphenyl) retinamide (4-HPR) is a synthetic retinoid that induces apoptosis in tumor cells. (-)-Epigallocatechin-3-gallate (EGCG) is a potent antioxidant abundant in green tea with anticancer properties. In the present investigation, we have studied the combined effect of 4-HPR and EGCG to induce apoptosis and to inhibit angiogenesis and tumor progression of human neuroblastoma malignant SH-SY5Y and SK-N-DZ cells in vivo. The cells were treated with 1 μM 4-HPR, 10 μM EGCG, or combination of both and subjected to MTT assay and TUNEL staining. Western blotting was performed for molecules involved in both receptor and mitochondria mediated apoptotic pathways. Angiogenesis and subcutaneous tumor growth studies were performed in immunocompromised mice. Treatment with combination of 4-HPR and EGCG resulted in more than 70% apoptosis and 80% inhibition in cell proliferation in both cell lines. Apoptosis was associated with induction of both receptor and mitochondria mediated pathways. Our in vivo angiogenesis studies demonstrated significant inhibition of neovascularization after treatment with either agent alone and complete inhibition after treatment with combination of both agents. Studies also demonstrated marked suppression of subcutaneous tumorigenesis and solid tumor formation after treatment with combination of 4-HPR and EGCG. In conclusion, our studies demonstrated that treatment with combination of 4-HPR and EGCG effectively induced apoptosis and inhibited cell proliferation, angiogenesis, and growth of neuroblastomas in vivo. Therefore, the combination of 4-HPR and EGCG could be used as a potential therapeutic regimen for effectively controlling the growth of human malignant neuroblastomas. This investigation was supported in part by the R01 grants (NS-57811 and CA-91460) from the National Institutes of Health (Bethesda, MD).

Introduction

Neuroblastomas are the most common extracranial, malignant solid tumors in childhood with a dismal prognosis. Neuroblastoma originates from immature neuroblasts of the peripheral (sympathetic) nervous system and usually arises in a paraspinal location in the abdomen or chest. It generally occurs in infants and very young children and is rarely present in children older than 10 years of age. About 50% of neuroblastoma tumors occur in children younger than two years old and 75% occur in children less than 4 years old. The etiology of neuroblastoma is unknown, but it seems unlikely that environmental factors are involved. A subset of patients inherits a genetic predisposition to neuroblastoma, which is mapped to the short arm of chromosome 16, and these patients usually have multifocal primary tumors that arise at an early age . Neuroblastoma accounts for 8-10% of pediatric cancers and 15% of the deaths attributable to malignant conditions in children. In most cases, neuroblastomas have already metastasized outside of the original site at the time of diagnosis. Toxic and severe side effects are significant in the current treatment regimens, and there is little room to modify the present therapy. Alternative treatment strategies with no toxicity and minimal side effects are therefore necessary to improve the survival rate.

Epigallocatechin-3-gallate (EGCG) is a potent anti-oxidant abundant in green tea. EGCG blocks the activation of EGF and HER-2 receptors. EGCG also inhibits telomerase and DNA methyltransferase, the enzymes involved in tumor cell immortality. EGCG has been shown to bind and inhibit Bcl-xL, the anti-apoptotic protein involved in tumor cell survival and promotes tumor cell invasion. There is also increasing evidence to show the anti-tumor properties of EGCG against glioblastoma, prostate, cervical, and bladder cancers. However, the effects of EGCG on neuroblastomas have not been investigated.

N-(4-Hydroxyphenyl) retinamide (4-HPR) is a synthetic retinoid that induces apoptosis in tumor cells. It is observed that 4-HPR increases reactive oxygen species (ROS), decreases mitochondrial membrane potential and induces apoptosis in several neuroblastoma cell lines. In the present investigation, we examined the combination effect of EGCG and 4-HPR to induce apoptosis and to inhibit angiogenesis and tumor progression in SH-SY5Y and SK-N-DZ neuroblastoma cells.

Figure 1

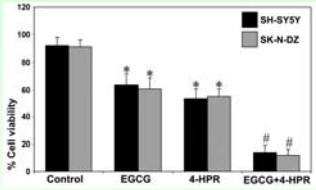

Figure 1. MTT assay for cell viability. MTT assay was performed in SH-SY5Y and SK-N-DZ cells after treatment with 10 μM EGCG or 1 μM 4-HPR or both together for 48 h. Data are mean ± SD of 6 independent experiments in duplicate (*p<0.001 compared to the control mean values and #p<0.001 compared to EGCG or 4-HPR mean values).

Figure 2

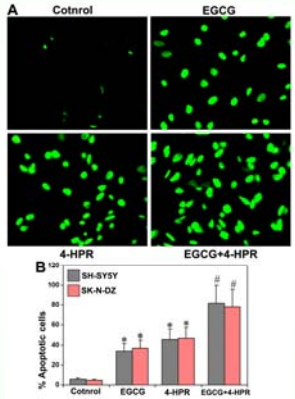

Figure 2. **(A)** TUNEL staining for detection of apoptotic cells after treatment with 10 μM EGCG or 1 μM 4-HPR or both together. The combination treatment resulted in a marked increase in apoptotic cell death in both cell lines than either treatment alone. **(B)** Quantitation of TUNEL-positive cells using Image-Pro Discovery software. Data are mean ± SD of 6 independent experiments in each group (*p<0.001 compared to the control mean values and #p<0.001 compared to EGCG or 4-HPR mean values).

Figure3

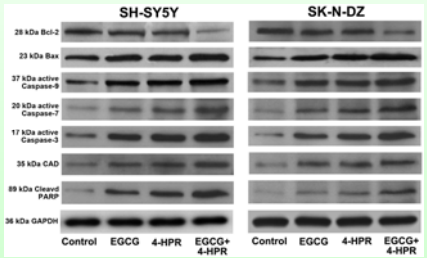

Figure 3. Representative Western blots for Bcl-2, Bax, active subunits of caspase–9, –7, –3, CAD, and cleaved PARP in the cell lysate of SH-SY5Y and SK-N-DZ cells after treatment with 10 μM EGCG or 1 μM 4-HPR or both together. Cleaved PARP was determined in the nuclear fraction. Mitochondria were isolated from the total cell lysate and cytochrome c levels were determined in the cytosolic fraction. An increase of cytochrome c levels in the cytosolic fraction indicates increased release of cytochrome c from mitochondria. Western blots were reprobed for GAPDH to demonstrate that equal amounts of proteins were loaded in respective lanes.

Figure 4

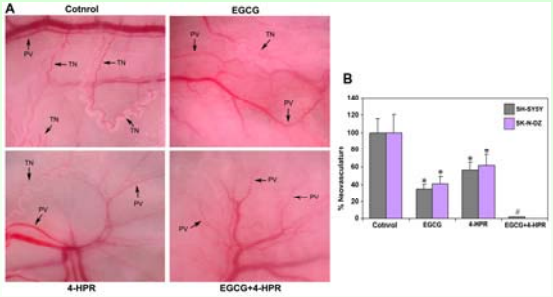

Figure 4. **(A)** In vivo angiogenesis assay. SH-SY5Y and SK-N-DZ cells treated with 10 μM EGCG or 1 μM 4-HPR or both agents together for 48 h were suspended in 200 μl of serum-free medium, injected into a diffusion chamber, implanted under the dorsal skin of nude mice and left for 10 days. Strong development of new microvessels (arrows, TN) arising from pre-existing vessels as curved thin structures in zigzag pattern was observed in control cells. The formation of such microvasculature was considerably reduced in both EGCG and 4-HPR treated cells and 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 ± SD of 6 samples from each group (*p<0.001 compared to the control mean values and #p<0.001 compared to EGCG or 4-HPR mean values). *TN: tumor-induced neovasculature; PV: pre-existing vasculature*

Figure 6

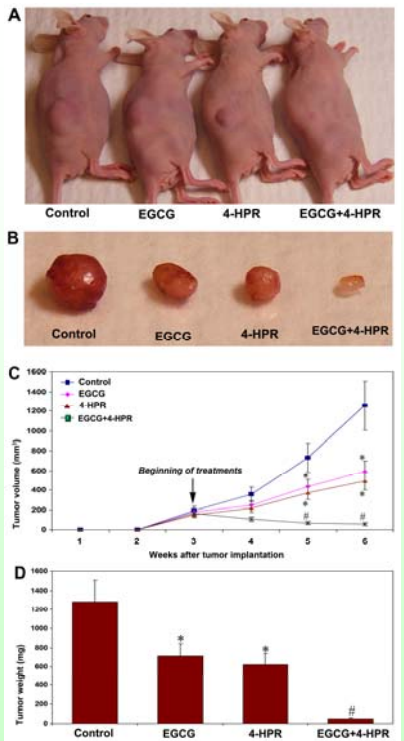

Figure 5

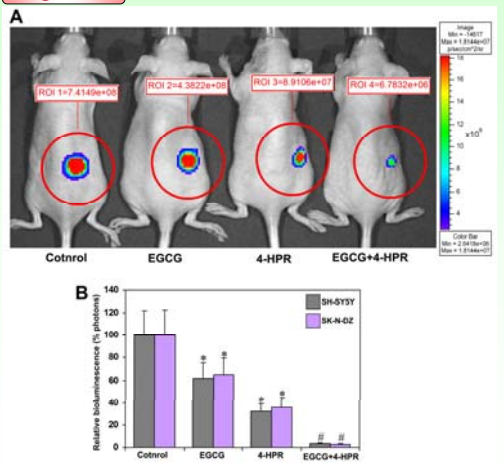

Figure 5. **(A)** Inhibition of subcutaneous tumorigenesis in immunosuppressed mice after treatment with EGCG and/or 4-HPR. SH-SY5Y and SK-N-DZ cells were stably transfected with luciferase gene and injected ($2x10^6$ cells) into the subcutaneous area of mice. Beginning from day 8, the mice were injected either with EGCG (10 μg/injection/mouse) or 4-HPR (1 μg/injection/mouse) or both agents together until day 20 on alternate days at the site of tumor cell implantation. On day 21, the mice were injected with luciferin and the resultant bioluminescence was visualized for the effect of treatments using Xenogen IVIS–200 imaging system. The data presented are representative of 6 sets of mice in each group. **(B)** Quantitative representation of relative bioluminescence as percentage photons after treatment with EGCG or 4-HPR. Data are mean ± S.D. of 6 mice in each group (*P < 0.001 compared to the control mean values and #P < 0.001 compared with EGCG or 4-HPR mean values).

Figure 6. **(A)** Inhibition of subcutaneous solid tumor development in nude mice after the treatment with EGCG and/or 4-HPR. SK-N-DZ cells were harvested, counted, suspended in an equal volume of high concentrated Matrigel and 100 μl of the suspension ($5x10^6$ 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 EGCG (100 μg/injection/mouse) or 4-HPR (10 μg/injection/mouse) or both agents together on alternate days for 3 weeks. At the end of 6th 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 EGCG and/or 4-HPR. Data are mean ± SD of 6 animals in each group. (*P<0.001 compared to the control mean values and #P<0.001 compared to EGCG or 4-HPR mean values). **(D)** Quantitative representation of tumor weight. Data are mean ± SD of 6 animals in each group (*P<0.001 compared to the control mean values and #P<0.001 compared to EGCG or 4-HPR mean values).

Conclusions

- ***Combination treatment with EGCG and 4-HPR resulted in decreased cell viability of both SH-SY5Y and SK-N-DZ neuroblastoma cells.***
- ***Combination treatment with EGCG and 4-HPR resulted apoptosis in more than 80% SH-SY5Y and SK-N-DZ neuroblastoma cells.***
- ***Combination treatment with EGCG and 4-HPR resulted in complete inhibition of tumor-induced neovasculature.***
- ***Simultaneous administration of EGCG and 4-HPR resulted in significant reduction of subcutaneous tumorigenesis in nude mice.***
- ***Combination treatment with EGCG and 4-HPR resulted in marked decrease of solid tumor development in the subcutaneous region of nude mice.***

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