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PHD THESIS

Influence of tyrosine kinase receptor and signaling pathways inhibition on cellular apoptosis in brain tumors

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INTRODUCTION

Cerebral tumors, divided into slow, benign tumors, or tumors with malignant rapid-progression , generally produce neurological signs and symptoms before specific diagnosis and treatment, patients with brain tumors having high levels of disability.

Glioblastoma(GB), first described in 1926 in Cushing's brain tumor classification, is the most common primary cerebral cancer among adults and one of the most aggressive malignant brain tumors. GB has an incidence of 1.26: 1 (male vs. female) with an average survival rate of 13-16 months after standard therapy involving maximum surgical resection, radiotherapy and chemotherapy with temozolomide. Unfortunately, the prognosis is unfavorable, with a survival rate of 5% at five years, with a minor increase of survival rate for patients diagnosed below the age of 20.

Despite new advances in drug discovery, most new specific therapies are still in clinical trials, both as single treatments or in combination with standard chemotherapeutics (temozolomide, carboplatin, cisplatin, lomustine) or radiotherapy.

I. CURRENT KNOWLEDGE

Brain tumors remain one of the major causes of cancer death, although a decrease in the incidence of intracranial tumors is observed worldwide. In 2008, the International Agency for Research on Cancer (IARC) estimated an increase in brain tumors in developed countries: 8 cases per 100,000 for men and 4.4 per 100,000 for women, with developing countries having a lower incidence of neoplasms: 3.2 per 100,000 for men and 2.8 per 100,000 for women.

Certain tumor biomarkers are studied in the context of their correlation with the occurrence and evolution of tumors, the most well-known being: vascular endothelial growth factor - VEGF, fibroblast growth factor - FGF, platelet growth factor - PDGF. The reaction of endothelial cells in the tumor vessels in adults is mediated by numerous tyrosine kinase receptors (PDGFRA, VEGFR1, VEGFR2, EGFR). Numerous studies have demonstrated overexpression of proangiogenic factors, including fibroblastic growth factor, vascular endothelial growth factor, endothelial platelet growth factor that

promotes endothelial cell proliferation and migration in primary cerebral tumors. Tumor apoptosis is induced by selective angiogenesis inhibitors, tumor proliferation, and inhibition of signaling pathways and is facilitated by caspase cascade activation. Caspases 2, 8, 9 and 10 are associated with the initiation of apoptosis signaling and receptor binding, called "death receptors" (DR). Caspases 3, 6 and 7 are studied for their role in regulating apoptosis and are known as caspases effectors. Angiogenesis is induced at the beginning of the stages of malignant tumor development, being pathologically promoted by a multitude of genetic modifications. Glioblastoma angiogenesis is characterized by microvascular proliferation around necrotic areas in response to the hypoxic environment, which in turn increases the expression of angiogenic factors and signaling pathways (RAS / RAF / ERK / MAPK, PI3K / Akt pathway and signaling cascade WNT).

A vast number of alternative methods of chemotherapy for brain tumors are currently being studied such as targeted therapies for angiogenic factors, growth factors and their receptors, or intracellular proteins that regulate growth, proliferation and invasiveness. New targeted molecular therapies are focused on angiogenic tyrosine kinase receptors (TRKs) and inactivation of their signaling pathways, and anti-angiogenic strategies are some of the most important in the clinical approach to molecular therapy.

A new method (metronomic method) for the administration of antiangiogenic therapy is characterized by lowering the dose of drugs and increasing the frequency of administration of the drugs. Some traditional medicines, such as cyclophosphamide and vinblastine, have had a significant antiangiogenic response. Moreover, when cyclophosphamide and bevacizumab was administered by the metronomic method , better results were observed than in the classical chemotherapy with bevacizumab.

Tumor monitoring after treatment is done with CT with contrast and MRI, new imaging response criteria should also be formulated for positron emitting tomography (PET). Also, tumor markers such as CD31, CD34, CD105, von Willebrand factor are used to monitor patient response to treatment.

Natural compounds extracted from plants are increasingly used in cancer therapy or as chemo preventive drugs. In fact, many of the anti-neoplastic drugs used in recent decades are either obtained directly from plants or are synthetic products derived from

certain natural structures. Medicines such as vinca alkaloids (e.g. vincristine, vinblastine, vinorelbine, vindesine) extracted from Catharantus roseus, taxanes (eg paclitaxel) extracted from the Pacific sheep tree bark have been shown to be some of the most effective anti-cancer drugs. Curcumin is a polyphenol derived from Curcuma longa, a rhizome also known as turmeric. Turmeric has been used since ancient times (over 2000 years ago) in Indian ayurvedic medicine for the treatment of a number of diseases, for example: various infections, burns, allergies, rheumatism, liver disorders, etc. Currently, researchers have discovered new curcumin properties such as antiproliferative, antimetastatic, antiangiogenic and anti-mutagenic properties.

II. PERSONAL CONTRIBUTIONS

MATERIALS AND METHODS

The GB9B and GB10B cell lines were obtained from tumor samples collected from patients diagnosed with glioblastoma at Bagdasar Arseni Hospital in Bucharest. After a small fragment of the tumor was triturated and filtered, the sample was centrifuged for 10 minutes, and the cells were resuspended and cultured using standard procedures. Cells were grown in cell culture flasks maintained in a 95% humidified air and 5% CO_2 incubator at 37℃. Cells were seeded in 96-well culture plates (3×10^3 cells / well) and treated with different concentrations of curcumin, SU1498 (a selective inhibitor of VEGFR2), AG1433 (a small molecule PDGFR inhibitor) and BEZ235 (a dual inhibitor of PI3K / mTOR) for three days. Apoptosis was analyzed using the ApoTarget Caspase-3 (CPP32) Colorimetric Protease Assay kit, ApoTarget Caspase-8 (FLICE) Colorimetric Protease Assay kit, ApoTarget Caspase-9 (Mch6 / Apaf-3) Colorimetric Protease Assay kit using the label recommendations.

The statistical analysis was performed using Microsoft Excel (Microsoft Corp., Redmond, WA, USA), XLSTAT and IBM SPSS Statistics 20.0 (IBM Corporation, Armonk, NY, United States). To test the normality of the data, the Anderson-Darling test was used. Variance analysis (ANOVA) and t test were used to analyze the significance of differences between study groups. Probability level values $p < 0.05$ were considered

statistically significant. All data is represented as mean ± standard deviation (SD). All experiments were done in three copies.

RESULTS

In this study, we used four GB9B glioblastoma cell lines. These are small passage GB cell lines established from tumor samples taken from patients diagnosed with glioblastoma after sowing the concentration of 2x104 GB9B glioblastoma cells in 6-well plates were plated in DMEM / F- 12 HAM for 72 hours.

We analyzed the effect of AG1433 (a small molecule inhibitor of PDGFR) on GB9B cells. To examine the effect of the drug on cell viability, exponentially increased GB9B cells were exposed to increasing doses of AG1433 (0.1, 1, 5, 10, 20, 30, 50, 60 and 100 μM) for three days, and the cytotoxic effect of the inhibitor was assessed by the MTT assay. The highest concentrations of AG1433 (100 μM) resulted in a significant decrease in cell viability at 50.3% at 48 hours and 56.5% at 72 hours.

We analyzed the effect of SU1498, a selective inhibitor of VEGFR2, on GB9B cells. We have observed that GB9B cells responded to treatment in a dose-dependent manner over time.

The doses of BEZ235 used in our experiments were much lower compared to those of AG1433 and SU1498. The best cytotoxic effect of the small molecule inhibitor was obtained at the concentration of 100 nM.

The caspase 3, caspase 8 and caspase 9 activity was investigated after treatment of GB9B cells with AG1433, SU1498 and BEZ235. Three hours after treatment of glioblastoma cells with AG1433, all three caspases were activated. At 8 hours after treatment, caspase 3 and caspase 8 remained active, while 24 hours after treatment, caspase 3 was still active. After treatment of GB9B cells with a small molecule VEGFR inhibitor as tyrosine SU1498, caspase 3 was activated three hours after administration and remained active at 8 hours and 48 hours after treatment. Caspase 9 and caspase 8 were activated only 48 hours after administration of SU1498. When we treated GB9B cells with BEZ235, a dual molecule inhibitor of the intracellular PI3K / Akt / mTOR signaling pathway, it was observed that caspase 3 and caspase 8 were activated 3

hours after treatment. At 8 hours and 48 hours after drug administration, all three caspases were activated while 24 hours after BEZ235, only caspase 3 and caspase 8 were active.

We evaluated how different tirofosin inhibitors (SU1498, AG1433 and BEZ235) induced apoptosis in the GB10B glioblastoma cell line by determining the caspase activity of 3, 8 and 9, knowing that caspase 3 is an executing protease, whereas caspases 8 and 9 are initiators of apoptosis. Thus, we evaluated caspase activities 3, 8 and 9 at 3, 8, 24 and 48 hours after treatment with SU1498, AG1433 and BEZ235 inhibitors. The caspase 3, caspase 8 and caspase 9 activity was evaluated after treatment of three glioblastoma cell lines (GB3B, GB4B, GB5B) with curcumin at 4 hours, 8 hours, 12 hours, 24 hours and 48 hours.

DISCUSSIONS AND CONCLUSIONS

One part of our study used two small glioblastoma cell lines: GB9B and GB10B, to determine the effect of some tyrosine kinase inhibitors. Using cell lines is the first step leading research into clinical trials. In fact, the most important preclinical studies investigating the cytotoxicity of specific drugs use standard cell lines. However, the results of these studies often do not correspond to in vivo observations. One of the causes could be that established tumor cell lines fail to reproduce tumor heterogeneity. Another reason is that all cell cultures of cancer cells found in high passage accumulate a series of mutations. Unlike standard cell lines, low passage tumor cell cultures are capable of preserving the phenotype and genotype of the original tumor. The proliferation rate of GB9B cells in the low passage was 0.3024 and the doubling time was 2.29 days.

In our study, glioblastoma cells responded in a time-dependent manner to inhibition of PDGFR by tyrosine AG1433. However, the drug was unable to induce more than 57% cytotoxicity, even at a concentration of 100 μM, which is reported to be very high. Thus, the cytotoxic effects of AG1433 on GB9B cells were modest, and the IC50 and IC50 values resulting from AG1433 treatment on the GB10B line were 1.8 and 67.4 μM, respectively.

Although AG1433 and SU1498 had a cytotoxic effect on the proliferation of GB9B cells, their antitumor activity was not as effective as expected. An explanation for this observation could be the co-activation of alternative tyrosine kinases that lead to the activation of other intracellular signaling cascades. These include phosphatidylinositol-3 kinase (PI3K) and protein kinase B (AKT) / mammalian rapamycin target (mTOR), both very important in gliomatogenesis by regulating cell growth and survival. It is already known that in nearly 70% of the glioblastomas the PI3K / AKT / mTOR intracellular signaling pathway is overactivated.

Then, we wanted to determine if the small molecule inhibitors used in our experiments were able to activate caspases 3, 8 and 9 in GB9B cells.

In fact, in recent years, drugs have been described that are capable of synthesizing caspases. These include small molecule activators of caspase. In this study, the ability of the small molecule AG1433, SU1498, and BEZ235, to activate caspases: 8, 9 and 3 on the GB9B cell line was determined. It was found that AG1433 activated all three caspases 3 hours after administration. After 8 hours, only caspase 3 and caspase 8 were still activated. The same effect was observed at 4 hours after treatment, while at 24 hours only caspase 3 was activated. Treatment with VEGFR inhibitor SU1498 only activated caspase 3 at three hours and at 8 hours, whereas after the drug administration for 48 hours, all three caspases were activated. Treatment of GB9B cells with BEZ235 at three hours and 24 hours after administration resulted in the activation of caspase 3 and caspase 8. At eight hours and 24 hours after drug administration, all three caspases were activated.

In our study it has been found that in vitro curcumin destroys glioblastoma cells in a dose-dependent manner. The results are consistent with other studies that have demonstrated the beneficial effect of turmeric treatment not only on brain tumors but also on other types of solid tumor cell lines such as: breast, colon, pancreas, lung

In this study three small cell passages isolated from glioblastoma tumors (GB3B, GB4B, GB5B) were used. The proliferation rate was approximately the same for all three cell lines. In fact, the GB3B cell line doubling time was 5 hours less than that of the GB4B cell line and 10 hours less than that of the GB5B cell line, but the difference between them was not statistically significant. All cell lines studied responded in a dose-

dependent manner to treatment. The lowest concentration of curcumin that induced cell death in cells of glioblastoma was 1 μM for all three cell lines.
In this study the ability of curcumin to activate caspases: 8, 9 and 3 on cell lines GB3B, GB4B and GB5B was determined. Curcumin has been found to activate caspases 3 and 9 at 4 and 8 hours, respectively, on all cell lines studied. After 8 hours, caspase 8 was activated on the GB4B line. Twelve hours after treatment with curcumin caspase 3, caspase 9 as well as caspase 8 are activated on all cell lines. Caspases 3 and 9 are active on all lines at 24 hours. The same effect was observed 48 hours after treatment.

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