SUMMARY OF DOCTORAL THESIS

The role of circulating progenitor stem cells
and growth factor receptors
in diagnosis and therapy of brain tumors

SCIENTIFIC COORDINATOR
Prof. Univ. Dr. Dricu Anica

PhD CANDIDATE
Băncioiu Mihai Daniel

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KEY WORDS: glioblastoma, circulating progenitor stem cells, growth factor receptors

GENERAL PART (STATE OF KNOWLEDGE)

Although the most recent cancer treatments control tumor cell growth and proliferation, they are not able to completely eradicate the tumor cell mass. The standard therapies for brain tumor pathology are represented by surgery, radiotherapy and chemotherapy. The inconvenient of these methods consists in the reduced specificity for tumor cells, besides significant side effects. In addition, they may cause further de novo tumors. Currently, the biology of brain tumors is not completely known, especially regarding the relationship with the adjacent parenchyma.

Brain tumors vary in type, location, growth rate and they are extremely heterogeneous in terms of phenotype and genotype. Glioblastomas are the most common brain tumors in adults. The grade IV glioblastoma have the highest grade of malignancy and it is the most aggressive and common form of brain tumour, characterized by intense cellularity, mitotic activity, microvascular proliferation and necrosis.
Due to the increased microvascular growth, a specific process for the high-grade malignant gliomas, it was concluded that the processes of angiogenesis and neoangiogenesis play an essential role in growth and invasion of glioblastoma. The release and the mobilization of the circulating endothelial progenitor cells (EPC) derived from bone marrow have an important role in neoangiogenesis.

A recently emerged concept is that tumor cells can produce various molecular signals that release and mobilize the circulating EPC produced by the bone marrow to the tumor site, where they stimulate the neoangiogenesis. The identification of EPC and the targeted therapy against them is a major challenge in modern anticancer therapy. EPC proved to be a potential marker for both tumor neoangiogenesis and for the anti-angiogenic therapy response. Because circulating ECP were reported to be elevated in various types of cancers, such as lung, breast, gastric, ovarian cancer, the attention was oriented towards the study of the circulating EPC level in glioblastoma. Thyrosin kinase growth factors and their receptors are thoroughly studied in cancer diagnosis and therapy. Inactivation of growth factor receptors might modulate tumor progression and response to chemotherapy and radiotherapy. In this study, we focused on the following factors: IGF-1, EGF, PDGF, VEGF and the corresponding receptors, known to be involved in stimulating cell growth, proliferation, invasion and inhibition of apoptosis.

The idea of this work was encouraged by the use of new therapeutic methods in clinical practice. Several therapeutic methods, such as gene therapy, immunotherapy (monoclonal antibodies, tumor vaccination), antisense oligonucleotides and growth factor receptors inhibitors, have shown potential useful clinical responses. Among the therapies that target these molecular changes, growth factor receptors inhibitors selectively inhibit receptors autophosphorylation and its activation. For this reason, in this study were investigated the cytotoxic effect of Gleevec (STI571), a small molecule growth factor receptors inhibitor, on glioblastoma cells in vitro. Therefore, the current study aims to investigate the potential of these transmembrane proteins as therapeutic targets in glioblastoma treatment.

SPECIAL PART (PERSONAL CONTRIBUTIONS)

AIMS:

1 Evaluation of the number of circulating ECP in patients with glioblastoma compared with patients diagnosed with low-grade brain tumors;
In vitro determination of the effect produced by the STI571 treatment on glioblastoma cells;

In vitro evaluation of the cytotoxic effect of SAM methylation agent on high-grade brain tumors (HGG) and its interference with the IGF-1R expression.

MATERIALS AND METHODS

The reagents were obtained from: Invitrogen/Life Technologies, Inc.(Rockville, MD, SUA), Sigma (St. Louis, MO, USA), Novartis Pharmaceuticals Corp. (Basel, Switzerland), R&D Systems (Abington, UK), Roche Diagnostics Gmbh (Mannheim, Germany), New England Bio-labs.

The cell line BT1GB was established from tumor biopsy collected from a glioblastoma diagnosed patient, surgically operated at the Neurosurgery Clinic of Bagdasar Arseni Hospital – Bucharest, following standard procedures. The primary 18 and 38 cell cultures used in this study were established from high-grade gliomas at the Academic University Hospital of Uppsala, according to standard procedures. The cell lines were cultured in MEM containing 10% fetal bovine serum (FBS), 2 mM glutamine and antibiotic (100 UI/ml penicilline and 100 UI/ml streptomycine). The cells were grown in tissue culture flasks maintained in a 95% air/5% CO2 atmosphere at 37°C in a humified incubator. Cells at the same passage ages were used for experimental purpose.

In this thesis we used the following experimental methods: survival fraction determination, Western Blot, MTT cellular proliferation assay, flow citometry.

In order to determine the dose-response curve, cells were grown in standard culture conditions and treated every 24 hours, for 3 days, with different dose of STI571, as follows: 0.3125 μM, 0.625 μM, 1.25 μM, 2.5 μM, 5 μM, 10 μM, 20 μM, 40 μM, 60 μM or 80 μM. The cell viability was analyzed after 72 hours. To determine the role of growth factors on the cytotoxic effect of STI571 treatment, the cells were incubated with 1% FBS with or without the addition of 50ng/ml IGF-1, PDGF-BB, VEGF-B, SCF, EGF or FGF-2, and treated with 1.25 μM, 2.5 μM, 5 μM or 10 μM STI551 every 24 hours, for 3 days. We included control groups in all experiments in the study.

Determination of cell viability

Cell viability was determined by MTT method or by daily counted of the cells from marked areas. The percentage survival of cells was then calculated by reference to the control.

Western blotting

Separation and detection of proteins was accomplished by Western Blot method. Briefly, the total cell lysate was electrophoretically separated on a 10% SDS-PAGE gel and the proteins were
transferred to a nitrocellulose membrane. After fixing and blocking with specific primary and secondary antibodies, the complex was visualized using the ECL system.

**Flow cytometry**

In order to identify and quantify the EPC, the suspended cells were fluorescent labeled and then were analyzed by flow cytometry. To quantify the circulating EPC by FACS (fluorescent activated cell sorting), peripheral blood was incubated with fluorescent conjugated antibodies against CD45, CD34, CD31, CD133 or VEGFR2. For each isolation procedure, the corresponding control markers were used. After filtering, the number of cells CD45-/CD31 + / CD34 + and CD45-/ CD31-/CD34- / CD133 + / VEGFR2 + was quantified and expressed as cells/ml of blood, using flow cytometer CyFlow SL and FlowMax software.

**Statistical analysis**

All data are represented as mean +/- SEM. Data were analysed using ANOVA t-test for analysis. P < 0.05 values were considered statistically significant.

**RESULTS**

1 Evaluation of the number of circulating EPC in patients with glioblastoma compared with patients diagnosed with low-grade brain tumors;

The study evaluated the circulating EPC values in peripheral blood collected from 12 patients with brain tumors: 4 patients with glioblastoma (grade IV WHO) and 8 patients with low grade gliomas (grade I WHO). Of these, 1 patient was diagnosed with meningothelial meningioma, 1 patient with transitional meningioma, two patients with microcystic meningioma, 1 patient with clear cell meningioma, two patients with meningioma and 1 patient with psammomatous meningioma. The patients were surgically operated and histopathological diagnosed at the Neurosurgery Clinic of Bagdasar Arseni Hospital - Bucharest.

Circulating EPC were defined as CD45-/CD31 + / CD34 + / CD133 + / VEGF R2 + cells. Of the total cells, the lymphocyte population was isolated first, then the CD45- population was selected by CD31 and CD34 marking. Finally, the CD133+ VEGFR2+ cell population was selected.

Our results show that the average numbers of the circulating EPC were as follows: 64.5±6.3 cells/mL for the meningothelial meningioma patient, 74±6.3 cells/mL for the transitional meningioma patient, 67.3±37.5 cells/mL for the microcystic meningioma patients, 89.5±5 cells/mL...
clear cells meningioma patient, 52.5±15 cells/mL for the meningioma patients, 49.5±11 cells/mL psammomatous meningioma patient and 144.75±56 cells/mL for the glioblastoma patients. The EPC levels were significantly lower (aprox. 3 fold) (p<0.05) in all patients diagnosed with low-grade tumors, compared with the levels found in patients with glioblastoma. However, no significant differences were observed between groups with low-grade tumors.

2 In vitro determination of the effect produced by the STI571 treatment on GB cells;

In the first set of experiments, we evaluated the dose-response relation for the treatment with STI571. Cell survival was determined at 3 days after the treatment. In BT1GB cell line, the cytotoxicity caused by STI571 treatment was dose-dependent. BT1GB cells had a survival percentage that steadily declined from 98% after the treatment with 0.3125μM STI571, down to 8.8% after the treatment with 40μM STI571. Above this concentration, the survival percentage decreased slightly to 7% after the treatment with 80μM STI571.

Then, the effect of the STI571 treatment was determined on glioblastoma cells grown in standard conditions with or without nutrients. The percent survival decreased steadily with the increase of the STI571 concentration in cells maintained under standard conditions, but the values were higher than those obtained for cells treated with STI571 and grown in medium supplemented with 1% FBS. For example, after the treatment with 10μm STI571, BT1GB cells grown in medium with 1% FBS, survived in a percentage of 32.2%, while those grown under standard conditions survived in the percentage of 59.2%.

To verify whether the cytotoxic effect of STI571 inhibition is mediated by IGF-1R, PDGFR, VEGFR, SCFR, EGFR or FGFR receptors, we studied the effect of IGF-1, PDGF-BB, VEGF-B, SCF, EGF or FGF-2 stimulation on the STI571 induced cytotoxicity in glioblastoma cell lines. Our results showed that survival of BT1GB cell grown in 10% FBS medium was 0.98 times higher than the survival of cells grown in 1% FBS medium. The stimulation with growth factors counteracts the cytotoxic effect induced by STI571 in glioblastoma cells.

No significant differences between the stimulatory effects of growth factors used in this study were observed.

3. SAM induced cytotoxicity in GB cells through a mechanism independent of IGF-1R

Previous studies have shown that S-adenosyl-methionine (SAM), the main donor of methyl groups in numerous biological processes, may induce cancer cell death by altering the DNA methylation profile. Other scientific studies suggest that SAM inhibits the mitogenic effect of growth
factors in cancer cells. Also, our previous studies demonstrate the importance of IGF-1R in survival and proliferation of GB cells.

In this study, we investigated SAM-induced cytotoxicity in two cell lines of glioblastoma (18 and 38). Cell cultures were exposed for 7 days at different concentrations of SAM and cell viability was assessed by counting viable cells in marked areas. After treatment with 0.1 μM SAM, cell line 18 showed a slight increase in cell viability within 4 days after starting the treatment. The cell proliferation increased by 7%, 17% and 5% in the second, third and fourth day of treatment.

The treatment with 0.1 μM SAM produces inhibition of cell proliferation and an insignificant cell death in cell line 18, 5 days after treatment. This effect persists until the end of treatment, with approx. 97% percentage survival. In the cell line 38, the cytotoxic effect of treatment with 0.1 μM SAM occurs after 7 days. In the first 6 days of treatment, the cell viability increased as follows: by 12% in the second day; by 19% in the third day, by 24% the fourth day, by 27% the fifth day and by 8% the sixth day.

The treatment with 200 μM SAM had an augmented cytotoxic effect in both studied cell lines. Cells belonging to 18 cell line had 91% percentage survival in the second day, 77% in the third day, 59% in the fourth day, 52% in the fifth day, 43% in the sixth day, and in the last day of treatment showed a decrease in cell viability down 32%. The 38 cell line had a percentage survival of 93% in the second day, 85% in the third day, 77% in the fourth day, 70% in the fifth day, 59% in the sixth day, and only 45% in the seventh day of treatment. To explain the observed cytotoxicity after treatment with different doses of SAM, we studied the possibility that SAM-induced cytotoxic effect might be caused by hypermethylation of the igf-1r oncogene and of the inhibition of the IGF-1R protein expression. IGF-1R is a tyrosine kinase receptor, encoded by the igf-1r oncogene, located on chromosome 15q25-Q26, which promotes oncogenic transformation, growth and survival of cancer cells (Yu et al. 2000). Surprisingly, the treatment with the highest concentration of SAM (200 μM), did not effect the IGF-1R membrane expression, the results suggesting that SAM-induced cytotoxicity in HGG cells is not related to modification of IGF-1R gene DNA methylation profile.

DISCUSSIONS

First Study. Due to increased microvascular proliferation that does not occur in low-grade gliomas, but is specific for malignant gliomas (particularly glioblastoma), the processes of angiogenesis and neoangiogenesis play an essential role in growth and invasion of glioblastoma (GB). Recently, ECP isolated from peripheral blood, drew attention as possible targets for cancer diagnosis and treatment. Data from various experimental and clinical studies have shown that the use of
angiogenesis inhibitors reduced tumor vascularization and progression. The recently emerged concept is that tumor cells can produce various molecular signals that can liberate and mobilize circulating ECP produced from bone marrow to the tumor site, where they stimulate neoangiogenesis. Circulating ECP were found to be significantly elevated in peripheral blood of cancer patients (lung, breast, gastric, ovarian) compared with normal individuals. The number of circulating ECP in our experiments was significantly higher in patients with high malignant brain tumors, compared with patients diagnosed with low-grade brain tumors. Our results suggest that circulating ECP can be a potential biomarker in GB, but also a future therapeutic target for new treatments of glioblastoma.

**Second Study.** Because tyrosin kinase factors and their receptors are constitutive active proteins or overexpressed in malignant tissue, they represent specific therapeutic targets in cancer tissues. Multiple scientific studies have shown that many glioblastomas overexpressed tyrosin kinase receptors such as EGFR, PDGFR, VEGFR, FGFR and SCFR. Using this concept, we hypothesized that tyrosin kinase receptors are considered specific therapeutic targets for glioblastoma treatment. STI571 is a small molecule inhibitor, used at this time in the treatment of various forms of cancer.

Our results show that STI571 induced cytotoxicity in BT1GB cells in a dose-dependent manner. The IC50 concentration was found to be approximately 12.5 μM. The cytotoxic effect induced by 1.25μM STI571 treatment was 35% higher in glioblastoma cells grown in 1% FBS supplemented medium, when compared with cells growing in 10% FBS supplemented medium. This difference was observed even at low concentrations of STI571.

It is assumed that the antitumor action of STI571 is achieved by Bcr-Abl, cKit oncprotein, and PDGFR inhibiting, thus the mechanism of action of this molecule is not yet completely known. In recent years, clinical and experimental studies have shown that cytotoxic effects of STI571 may be influenced by the growth factors. It is already known that brain tumors secrete a variety of growth factors that stimulate proliferation, survival, migration and tumor angiogenesis through a paracrine or autocrine action. Our results show that stimulation with IGF-1, PDGF-BB, VEGF-B, SCF, EGF or FGF-2 growth factors increased proliferation of glioblastoma cells by about 0.5 fold. Low molecular weight inhibitor STI571 proved to be more effective in the treatment of glioblastoma in the absence of nutrients. The STI571 concentration that cause 50% cytotoxicity in cells growing in medium without nutrients is 4 fold lower (approximately 2.5 μM STI571) than that in cells growing in the standard conditions. The cytotoxic effect induced by 2.5 μM STI571 was reduced by 7.4% after stimulation with IGF-1, by 8.8% after stimulation with PDGF-BB, by 6.9% after stimulation with VEGF-B, by 12.8% after stimulation with SCF, by 7.1% after stimulation with EGF, and by 7.5% after stimulation
with FGF-2. The stimulation with growth factors had the effect of reducing the cytotoxic effect of treatment with STI571.

**Third Study.** Previous studies have shown that S-adenosyl-methionine (SAM), the main donor of methyl groups in numerous biological processes, may induce cancer cell death by altering the DNA methylation profile. Other scientific studies suggest that SAM inhibits mitogen effect of growth factors in cancer cells. Also, our previous studies demonstrate the importance of IGF-1R in glioblastoma cell survival and proliferation. The SAM-induced effect that we investigated was cytotoxic for both glioblastoma cell lines (18 and 38). We also analyzed the possibility that SAM-induced cytotoxicity was caused by inhibition of IGF-1R protein expression due to hypermethylation effect of *igf-1r* gene. The results of the present study, showed that SAM induces cytotoxicity in glioblastoma cells, but does not affect membrane expression of IGF-1R, suggesting that SAM-induced cytotoxicity is not related to the changes in the IGF-1R gene DNA methylation.

**CONCLUSIONS**

**First Study.** In the first part of the thesis, we determined and compared the levels of circulating ECP in peripheral blood in patients with brain tumors of varying degrees. The results lead to the conclusion that the number of circulating ECP is significantly higher in patients with high malignant brain tumors compared with patients diagnosed with low-grade brain tumors. However, no significant differences were observed between groups with low-grade tumors. These results suggest that circulating ECP can be a potential biomarker in glioblastoma, and also that ECP may represent a therapeutic target for new future treatments of glioblastoma.

**Second Study.** Here, we investigated the potential role of STI571 in the treatment of glioblastoma. Our results showed that in BT1GB cells, the small molecule inhibitor STI571 induced cytotoxicity in a dose-dependent manner. The cytotoxic effect induced by the treatment with 1.25 μM STI571 was 35% more effective in the glioblastoma cells grown in 1% FBS supplemented medium, compared with the glioblastoma cells growing in 10% FBS supplemented medium.

The cytotoxic effect of the treatment with STI571 was reduced by growth factors (IGF1, PDGF-BB, VEGF-B, SCF, EGF, FGF-2) stimulation in glioblastoma cells *in vitro*

**Third Study.** In the last study we evaluated the effect of SAM methylation agent on high-grade brain tumors (HGG) and its interference with IGF-1R expression. We concluded that SAM induces cytotoxicity, but does not affect the IGF-1R membrane expression, suggesting that SAM-induced cytotoxicity is not related to the change in IGF-1R gene DNA methylation profile.