Short thesis for the degree of Doctor of Philosophy (PhD)

CONNECTION OF THE EXTRACELLULAR MATRIX COMPOSITION WITH BOUNDARIES IN THE PRESENT THERAPY OF GLIOBLASTOMA

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The Examination takes place at the Library of the Department of Neurosurgery, Faculty of Medicine, University of Debrecen, on 9th September, 2016 at 11:00 am.

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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medcine, Faculty of Medicine, University of Debrecen, on 9th September, 2016 at 13:00 am.

1. INTRODUCTION

After leukaemia, central nervous system (CNS) malignancies are the second most common cause of death due to tumorous disease among males between the ages of 20 and 39. Moreover they are the most common tumor type among children under the age of 19. Basicly we distinguish two groups: primary (de novo development in the CNS) and secondary (metastatic) type neoplasms. Among primary lesions gliomas have a pivotal role according to their morbidity and mortality. Gliomas are tumors originating from the neuroepithelial tissue which contains neurons and their supportive glial cells: astrocytes, oligodendrocytes, ependymal cells, radial glias, microglias. Malignant transformation of glial cells results in the formation of different types of gliomas. The World Health Organization grades these tumors on I–IV scale according to their appearance, recurrence potential, treatment and degree of aggressiveness. Grade I-II comprise low grade gliomas (LGG) while grade III-IV stand for high grade gliomas (HGG).

Grade IV corresponding to the most malignant type, that is glioblastoma multiforme (GBM) which has the greatest significance among HGGs. Responsible for 54 percent of gliomas and 17 percent of all primary CNS malignancies, such tumors are rapidly progressing, aggressive and have an extremely poor prognosis.

In the past decades surgical resection alone resulted in a disappointing median survival of 3-5 months. Later postoperative radiotherapy prolonged the survival to 8-11 months. Numerous chemotherapeutic agents were also tested, applied alone or in

combination with radiotherapy, but significant improvement in life expectancy could not be achieved for a long time.

In 2005 Roger Stupp and his colleagues published the promising results of conformal irradiation in combination with temozolomide chemoterapy in 576 patinets, which increased the survival rate to median 14,6 months. Later this study was proved to be a cornerstone in the postoperative treatment of GBM and since its introduction, the concurrent radiochemoterapy (also called the ’Stupp’ protocol) has become the ’gold standard’ treatment.

Temozolomide is an alkylating agent from the imidazotetrazine family with favourable pharmacokinetic features, good CNS penetration rate, a nearly 100 percent biological accessibility and its use causes relatively mild side effects. Temozolomide alkylates the DNA of the tumor cells, which leads to single- and double-strand breaks performed by DNA repair enzymes, which in turn results in activation of apoptosis pathways. Temozolomide is the first choice drug in newly diagnosed GBM and its positive effect has been proved in other gliomas as well. At first, in combination with irradiation it is used at 75mg/m2 dose in the concurrent phase, after that it is used at 150mg/m2 and subsequently at 200mg/m2 dose as monotherapy. The adjuvant effect in concurrent therapy is due to the radiosensitization of temozolomide, which amog other things can be achieved through the following mechanisms: repairing insufficency of the damaged DNA, autophagy, apoptosis induction and reversal of epithelial-mesenchymal transition.

Despite complex and intense treatment, recurrence is inevitable and causes relatively rapid death. By now, GBM is irreversible, and the treatment goal is to delay progression in order to preserve the patient's quality of life as long as possible [3]. The main cause of repeated recurrence is the tumor's aggressively infiltrative nature, which makes radical surgical resection impossible. The most radical resection possible and subsequent radio- and chemotherapy are the fundamentals of the treatment.

On the other hand metastases vastly differ from gliomas in their appearance, behavior and treatability. They have a general incidency between 9% and 17%, but it is increasing from year to year due to the continuously developing imaging techniques and the increasingly effective systemic oncotherapies. The new therapies prolong the patinets' survival giving tumors the opportunity to form CNS nests. The most frequent types of primary source are lung cancer, mammary cancer and melanoma, they add up to 80% of all cases. Among them lung cancer has the most significant role bearing 30 to 60% alone. Patients' life expentancy is pretty much higher with soliter and oligo metastases in contrast with GBM and the prognosis depends on the primary source. Treatment of a secunder brain tumor can be surgical removal or irradiation. When it comes to radiotherapy there are different types available: whole brain / focal brain radiotherapy, 3D conformal radiation and stereotactic methods. Surgical treatment highly depends on the size and location of the metastasis and its relation to the surrounding brain. In general a metastatic tumor mass usually has well-defined borders, it does not infiltrate or stick to the surrounding tissue thus the total removal of a single intracerebral metastasis is in general a routine neurosurgical procedure.

Primary and secondary CNS tumors make contact with the nearby brain tissue highly differently which specify their spreading and thereby the success of their oncotherapy. The highly invasive malignant gliomas strongly differ from the spheroid cerebral metastases and their infiltration zone can barely be differentiated intraoperatively from the peritumoral intact brain tissue, which make their radical resection impossible in most cases. ECM has probably the prominent role in this varied invasive behaviour.

Cells of the central nervous system, similar to cells of any other organ, are surrounded by a protein- and fibre-rich substance called the extracellular matrix (ECM).

Regarding its components, the matrix is fairly heterogeneous, containing collagens, elastin, laminins, fibronectin, proteglycans, glycosaminoglycans, enzymes, etc. The different molecules, enzymes and soluble factors are embedded in a network of connective fibre strands. The extracellular matrix represents a dynamic and active environment to the cells. A strong connection is established between the cells and the sorrounding matrix in this environment which plays a crucial role in local distribution of malignancies through secondary signal transduction pathways. Invasion of tumor cells requires close connection with the ECM. This cell-ECM connection with the cell – cell interaction and the system of the soluble factors – establishes a complex communication network. Invading tumor cells induce definite changes in the expression of the matrix components and in the activities of proteases and synthases. These peritumoral changes in the ECM are responsible for the largely different invasive behavior of the gliomas and the intracerebral metastases. To understand the fairly modified invasive potential of anaplastic intracerebral tumors of different origin,

the effect of tumor on the peritumoral ECM and alterations of invasion related ECM components in the peritumoral brain should be evaluated. The understanding of this and the mapping of the alterations can provide us with useful informations about the distinct behavior of the different intracranial tumors and the concept of developing an effective new oncotherapy.

2. OBJECTIVES

Levels of different ECM molecules in intracranial tumors has already been measured in the literature, but only a few of the research groups has done panel- like studies recently to establish an expression pattern. In addition, the peritumoral tissue that surrounds the brain tumors is a barely unknown field of research in neuro-oncology and we have insuffitient knowlwdge on the impact of postoperative chemoradiation of the GBMs from the ECM ponit of view. Considering these facts our work is actually pioneer in this field.

Nevertheless, there are great efforts to explore new target-based agents that can solve the problem of inevitable recurrence but the real success has not arrived yet. The target of many of these potential therapies is the extracellular matrix (ECM), because peritumoral infiltration of glioma cells is regulated mainly by ECM molecules (collagens, proteoglycans, laminins, hyaluronan, and synthesizing and degrading enzymes), which create an active dynamic medium. Alteration in ECM composition

plays a key role in cell movement, which can be observed by determining the expressional changes of the relevant molecules. Once the oncotherapy alters the expression of ECM molecules, it may affect the infiltrative potential of the tumor. The reduction of the tumor invasion activity from the concurrent treatment can not be expected without this and subsequently there won't be any hope for a radical surgical removal after oncotherapy.

The question then arises whether the invasion induces any molecular change in the peritumoral tissue, it differs in primary and secondary brain tumors or not and the present standrad treatment protocol for glioblastoma can cause any alteration in the tumor ECM, in other words can the concurrent therapy infulence the infiltration? In order to answer these questions we aimed two objectives:

- to examine the ECM components and the adjacent molecules from intracerebral tumor tissue and non-tumor brain tissue that play role in peritumoral invasion.
- to determine the expression pattern of ECM molecules in GBM tissues before and after oncotherapy and perform comparative analyses.

3. METHODS

The first step to examine the differencies and the alterations of the matrix molecules due to various agents in the CNS was to collect and store human brain samples.

Therefore in december, 2005 we established Neurosurgical Tumor and Tissue Bank of Debrecen. We stored the intraoperatively removed and fresh frozen samples taken from patients with prior agreement at the Neurosurgery Department of the University of Debrecen. At present there are 55 different CNS lesions and samples from 1300 different patients in the tissue bank which are immediately frozen at the time of removal on the surface of liquid nitrogen and stored at -80°C until processing. We record many datas (patient identification, age, time of removal, verificated histological diagnose, sample numbers) that allow easy search and immediate statistical analysis.

At first, twenty-seven samples of human brain tissue were processed, which had been collected from different patients during neurosurgical intervention. Three different tissue groups were created: histologically verified peritumoral area of glioblastoma (peri-GBM), peritumoral area of intracerebral metastasis of bronchial adenocarcinoma (peri-Met), and non-tumoral brain tissue (Norm). Each group contained nine different samples. Peritumoral tissue samples were taken only if it was inescapable by tumor removal. Peritumoral samples were all collected from the tumor proximate area, which was considered intact by the surgeon intraoperatively. The tissues were further examined and approved by an experienced neuropathologist, excluding samples that were taken from the tumor stroma. The non-tumoral brain samples were collected during functional epilepsy neurosurgery and were completely free of tumor cells. After that thirty-one human brain tumor samples removed during neurosurgical operation were tested. The fresh frozen tissue samples were also selected from the Neurosurgical Brain Tumor and Tissue Bank of Debrecen; fifteen samples originated

from patients with newly diagnosed GBM without any oncotherapy, and sixteen samples were excised from recurrent tumors after chemoradiotherapy. The average size of the samples were $5mm^3$.

Postoperatively the so called 'Stupp' protocol means 60 Gy of conformal irradiation to the surgical site and to the safety zone, and parallel to this a daily dose of 75mg / m^2 temozolomide given orally. After the concurrent phase the administrasion of 150 mg/m^2 in the first cycle then 200 mg/m^2 temozolomide alone as monotherapy (5 day treatment in 28 day cycles).. Each tissue sample was approved by an experienced neuropathologist as well for further investigation. All procedures were approved by the National Ethical Committee of Hungary and every patient signed an informed consent form before surgery.

The final concentration of ECM molecules in brain tissues is influenced by numerous processes through transcription and translation (e.g.: alternative splicing, posttranslational modifications), therefore we analyzed the matrix components at RNA (mRNA) and protein levels to create the expression profiles. First we focused on approximately 100 molecules which were selected based on literature data. According to our preliminary results we shortened the list progressively. Finally the role of the following 20 moelcules proved to be essential: brevican, N-cadherin-2, collagen type III- alpha1 chain, EGFR (erbB1), erbB2, fibronectin, CD168, integrin alpha1, alpha3, alpha7, beta1 chains, laminin alpha4, beta1 chains, matrix metalloproteinase-2 (MMP-2), matrix metalloproteinase-9 (MMP-9), neurocan, syndecan-1, tenascin-C, tenascin-

R, versican. We did not measure the syndecan-1 level in the peritumoral and non.tumor samples.

mRNA analysis

The first step in isolating RNA content from the tissues was the mechanical pulverization of the brain sample, then we added the right amount of Trizol. The instant homogenization of the pulverized sample in TriReagent was done by rotor-stator homogenizer. After centrifuging the right amount of chloroform was added to the supernatant. At the end of the second centrifuging the phases separated: the upper aqueous phase (containing the RNA), interphase (containing proteins) and the lower phenol phase (containing the DNA). The RNA layer (which was important for us) was dissolved in nuclease free water. After complete dissolution the quantity, purity and quality of the isolated RNA were assessed. We used RNEasy for purification, and the purified RNA samples were converted to single-stranded cDNA using reverse transcription polymerase chain reaction. TaqMan Low Density Array (TLDA) experiments were performed using the Applied Biosystems 7900HT real-time PCR system with the Micro Fluidic Card upgrade. The Micro Fluidic Cards were analyzed with the SDS 2.1 software as relative quantification studies and the CT values were exported for further analysis. β-actin and glyceraldehyde 3-phosphate dehydrogenase housekeeping genes showed the least variation among the samples and were used as the reference genes to calculate the dCt value for each gene. Expression values were calculated using the comparative CT method.

Protein analysis

Tissue homogenisation for protein analysis was performed as described in the case of RNA purification, but a lysis buffer containing 50 mM Tris, 1mM EDTA, 17 mM beta-mercaptoethanol and 0.5% Triton-X100 was used in this case for tissue lysis. The protein content was measured using the Bradford method and equal amounts of proteins were used for in-solution trypsin digestion. The selected reaction monitoring (SRM)-based targeted proteomic method was developed for relative protein amount determination. For protein concentration estimation, the area under the curve of the acquired spectra was calculated; those SRM spectra were used for AUC calculations where the intensity of the signal exceeded 500 cps. The data integration was done with the help of the Analyst 1.4.2 software based on the curve shape determined from pilot analyses.

Statistic analysis

To confirm presumed differences among the three groups we used ANOVA test when the requirements were met, in other cases Kruskall-Wallis test were done. After that we performed Mann-Whitney U-test to compare two sample groups and identify genes and proteins with significantly different expression levels. Significant differences were established at $p < 0.05$. Expressional changes were further analysed by calculating 95% confidence intervals (CI).

4. RESULTS

mRNA-expression

First of all twenty-seven samples containing nine peri-GBM, nine peri-Met tissue samples originating from the peritumoral area of glioblastoma and cerebral metastasis and nine normal samples were investigated. The expression levels of the two different peritumoral tissue groups were compared to each other and to the normal tissue. Focusing on the peri-GBM versus peri-Met comparison, ten molecules (brevican, ErbB1, ErbB2, integrin alpha-1, integrin alpha-3, integrin alpha-7, laminin alpha-4, laminin beta-1, tenascin-R, versican) showed relative overexpression in peri-metastatic tissue, while mRNA overexpression in the peritumoral area of glioblastoma was observed in nine genes (N-cadherin-2, collagen alpha-1, fibronectin, CD168, integrin beta-1, MMP-2, MMP-9, neurocan, tenascin-C) compared to peri-Met. We found statistical significance in two cases; both tenascin-C (95% CI: 7.95-12.92) and CD168 (95% CI: 2.75-4.64) were significantly overexpressed in peri-glioblastoma.

Overexpression in the glioblastoma adjacent tissue (peri-GBM) versus the normal samples (Norm) was observed in fourteen cases (N- cadherin-2, collagen alpha-1, erbB1, erbB2, fibronectin, CD168, integrin alpha-1, integrin alpha-3, integrin beta-1, laminin beta-1, MMP-2, MMP-9, neurocan, tenascin-C), but it was statistically significant only in the case of CD168 (95% CI: 0.30-0.44). Five genes (brevican, integrin alpha-7, laminin alpha-4, tenascin-R, versican) were under-expressed in peri-

GBM in comparison to Norm. Only Tenascin-R (95% CI: 1.40-5.11) was significantly lower in the peri-GBM samples than in the normal brain tissue.

The mRNA level of thirteen molecules (brevican, collagen alpha-1, erbB1, erbB2, CD168, integrin alpha-1, integrin alpha-3, integrin alpha-7, integrin beta-1, laminin alpha-4, laminin beta-1, MMP-9, versican) were elevated and only six (N-cadherin-2, fibronectin, MMP-2, neurocan, tenascin-C, tenascin-R) were depressed in peri-Met in comparison to the normal samples; nevertheless, no significance was detected.

The comparison of gene expression in treated and untreated samples showed that the mRNA level of twelve of the examined nineteen molecules exhibited underexpression after treatment: brevican, collagen type III alpha-1, fibronectin, intergrin alpha-1, integrin alpha-7, laminin alpha-4, laminin beta-1, matrix metalloproteinase-9, neurocan, syndecan-1, tenascin-R, and versican. Increased expression was detected in the case of seven molecules: cadherin-N2, CD168, erb-B2, integrin alpha-3, integrin beta-1, matrix metalloproteinase-2, and tenascin-C. The underexpression of matrix metalloproteinase-9 was significant (CI: 0.13 – 0.26).

Protein levels

After determining mRNA expression levels, we performed a label free SRM-based quantitative protein analysis to gain information from the translational phase to check the manifestation of the alterations at mRNA level [20, 21] (Figure 3). We could determine every protein of the examined genes except ErbB2, syndecan-1 and tenascin-C.

In the peri-GBM versus peri-Met comparison, the amount of eleven ECM-related proteins was higher in glioblastoma adjacent tissue, from which five showed concordant elevation with the mRNA levels (N-cadherin-2, CD168, integrin beta-1, MMP-9, neurocan). The elevation of CD168 in peri-GBM was significant (95% CI: 325.9-1109.1), as was the overexpression at the gene level. Six proteins were elevated around metastatic tissue, three of them showed concordance (integrin alpha-1, tenascin-R, versican). The elevation of fibronectin in peri-Met was significant (95% CI: [-642.5]-[-10.5]); however, it was contrary to the mRNA under-expression.

The level of thirteen proteins was higher in peri-GBM compared to Norm tissue, with ten showing concordance with the expressed genes (N-cadherin-2, collagen alpha-1, ErbB1, fibronectin, CD168, integrin alpha-1, integrin alpha-3, integrin beta-1, laminin beta-1, MMP-9). Only four protein concentrations were higher in Norm; two of them were concordant (tenascin-R, versican). No significance was detected.

Comparing peri-Met to Norm, eight proteins were higher around metastasis with seven concordant changes (collagen alpha-1, ErbB1, integrin alpha-1, integrin alpha-3, integrin alpha-7, integrin beta-1, versican). Similar to the peri-GBM–peri-Met comparison, the elevation of fibronectin in peri-Met was significant, although the level of mRNA was under-expressed. In the case of nine proteins, a decreased concentration was measured in peri-Met, with four showing concordance (N-cadherin-2, MMP-2, neurocan, tenascin-R) but no significant decrease.

The quantitative protein analysis showed that the level of twelve proteins decreased in the post-treatment samples: brevican, cadherin-N2, CD168, collagen type III alpha-1,

integrin alpha-3, integrin alpha-7, integrin beta-1, laminin beta-1, matrix metalloproteinase-2, matrix metalloproteinase-9, neurocan, and tenascin-R. Protein concentration increased after treatment in the case of five molecules: erb-B2, fibronectin, integrin alpha-1, laminin alpha-4, and versican. Syndecan-1 and tenascin-C could not be detected in the post-treatment samples. After oncotherapy, the level of brevican exhibited a significant decrease (95% CI: -8857.43 – -7.46).

The analysis of the expressional data at both RNA and protein levels detected concordant changes in eight cases. Consequent post-treatment underexpression was seen in the case of brevican, collagen type III alpha-1, integrin alpha-7, laminin beta-1, matrix metalloproteinase-9, neurocan, and tenascin-R. In contrast, the transcription and translation of erb-B2 was lowered after oncotherapy. The statistical analysis indicated only two significant changes: matrix metalloproteinase-9 at the RNA level (fold change: 0.21 p value: 0.006 95% CI: [0.13] - [0.26]) and brevican at the protein level (prot. level:-4432,44 p value: 0.006 95% CI: [-8857,43] - [-7,46]).

5. DISCUSSION

The ECM has been the subject of numerous researches recently, hence we have plenty of new information about its structural details, properties and important role in various physiological and pathological processes. For a long time it was considered that the ECM has only some structural functions, but it became clear step by step that it is

essential for the fuctional contact between the cells and their environment. It has been also proved that the ECM components have a crucial role in the management of different embryological processes, cell maturing, differentiation and migration, cell survival, maintain of the tissue homeostasis, invasion of tumor cells, etc. Any kind of inherited or acquired structural defect in the matrix causes cellular and tissue changes that result in worsening of existing diseases and the development of new ones. For example production of impaired laminin induces malfunctioning muscle structures that lead to muscle dystrophies. Despite the fact that ECM in the CNS has a lot of similarities to ECM in other tissues, clear differences can be discovered in the molecular composition, even some components can only be found in the brain. For instance the cerebral matrix has a relatively low concentration of fibrous proteins and large amount of glycosaminoglycans. Near the cell membrane of the neurons the matrix becomes more dense and forms basal membrane that consists of collagens, glycoproteins (especially tenascins), chondroitin sulfate and heparane sulfate proteoglycans (versican, neurocan, aggrecan, neuroglycan-C, perlecan, agrin), hyaluronic acid and cell adhesion molecules. Cadherins, elastin, CD44 cell membrane receptor, matrillin, syndecans, aquaporins, agrin, TGFβ and matrix metalloproteinases have also essential roles. Besides its important function in embryogenesis the ECM is crucial for tumor progression. Invasive behavior of the neoplasms and infiltration of the surrounding tissue require cell-cell and cell-ECM interactions.

In our study, invasion-related ECM-components and transmembrane receptors were tested in the peritumoral area of primary (glioblastoma), and secondary

(adenocarcinoma metastasis) brain tumors and in their infiltrative brain parenchyma before and after standard oncotherapy. Due to the analysis of the mRNA expression pattern and quantification of the protein levels, some of the ECM compartments and receptors seemed to correspond with the extent of the peritumoral invasion. Besides the few molecules that statistically significantly differed in the investigated tissue groups, some showed concordant alterations at both mRNA and protein levels. We found obvious differences in the peritumoral tissues when compared to each other and to normal brain tissue.

5.1. Peritumoral zone of glioblastoma

Tenazcins and CD168

Tenascins compose a large extracellular matrix glycoprotein family of five known members (tenascin -C, -R, -X, -Y, -W). Among them, with different alternatively spliced isoforms, tenascin-C (TNC) plays a key role in embryogenesis, wound healing and tumor progression. Tenascin-C was isolated from numerous embryonal and mature tissues e.g. epithelial tissue, smooth muscle cells and different tumors. This molecule contacts integrins, collagens, proteoglycans and fibronectin and can serve as adhesive or anti-adhesive protein according to the cell environment. In solute form it blocks the adhesion and migration of fibroblasts on fibronectin coated surface. During embryogenesis tenascin-C can be detected first which later vanishes in parallel with the appearance of tenascin-R. Tenascin-C can usually not be detected in normal mature brain, but can be found after injury or in basal lamina of tumors cells, where it

impedes connection between fibronectin and syndecan-4 thus promoting tumor progression and formation of metastases. In malignant gliomas, TNC has been shown to increase the invasiveness of glioma cells in an autocrine manner inducing a reactive change in the surrounding brain tissue. In addition, Hirata et al found that TNC expression was correlated with the volume of peritumoral reactive change in magnetic resonance images and with the prognosis of glioblastoma patients. Herold-Mende et al revealed that TNC level increased with tumor malignancy. Another family member, tenascin-R (TNR), has been implicated in the developing central nervous system, during regeneration and in a variety of cell-matrix interactions e.g. tumor cell adhesion and migration. Overexpression of tenascin-R was observed in pilocytic astrocytomas versus glioblastomas at both mRNA and protein levels. Accordingly, TNR expression was found to decrease with malignancy in astrocytic tumors.

CD168 (also known as HMMR, RHAMM) hyaluronan receptor affects cell migration and signalling, promoting metastasis and angiogenesis. Interacting with actin, calmodulin, microtubules and other mitosis-associated structures, the expression of CD168 is up-regulated in aggressive cancers.

Regarding peri-glioblastoma samples, most of the examined molecules were not significantly altered, with the exception of the overexpressed tenascin-C and CD168 and the underexpressed tenascin-R. This suggests that the ECM of the adjacent brain tissue of glioblastoma does not react definitely to the tumor. This might be the possible explanation why ECM cannot remarkably inhibit the invasion of the tumor cells into the peritumoral area. The results also underpin the role of these three

molecules in glioma cell invasion. We found significant down-regulation of the tenascin-R gene in peri-glioblastoma, suggesting that its decreased expression is a sign of malignancy and invasion potential. Thus TNR could impede peritumoral invasion, while TNC may facilitate it.

MMP-9

Enzymes also have a great impact on peritumoral invasion. The matrix metalloproteinase family can degrade and modulate different ECM components and cell surface receptors remodelling the matrix, promoting cell signalling and contributing to tumor progression. Focusing on the gelatinase group of MMPs, according to Veeravalli, MMP-9 has a great impact on glioma cell migration and invasion. In our study, MMP-9 is greatly up-regulated in the peritumoral area of glioblastoma both at the mRNA and protein levels, so it is supposed to facilitate tumor invasion to the peritumoral tissue, though significancy could not be detected.

5.2. Peritumoral zone of metastasis

Fibronectin

ECM glycoprotein dimer fibronectin binds to integrin cell receptors and other matrix components (fibrin, collagen, etc.). Helping cell adhesion, migration and differentiation, it plays a crucial role in embryogenesis, wound healing, tumor progression and metastasis formation. In gliomas, fibronectin expression tends to increase, promoting cell migration and invasion with a positive correlation to tumor

grade. On the other hand, according to Sabari et al fibronectin matrix assembly can play a suppressive role in glioblastoma cell dispersal. We detected significantly lower protein levels in peri-GBM than in peri-Met, suggesting a possible role of fibronectin in hindering tumor cell migration in the surrounding brain tissue.

The molecular changes that are induced in the metastatic peritumoral area can probably decrease tumor infiltration, presuming a peritumoral "mesh" around the metastatic tumor tissue that can inhibit the spread of tumor cells. The conspicuously increased level of fibronectin around metastatic tumor may contribute to the establishment of metastasis in an early step, helping the metastatic cells to attach, settle down and form tumor nests. This hypothesis is parallel to Paget's "seed and soil" concept. The lack of mRNA overexpression with the high protein levels suggests a secondary concentration of fibronectin - maybe from tumor origin - which needs further investigation.

The examination of peritumoral ECM in case of intracerebral tumors could identify molecules that probably (negatively or positively) affect the invasion processes.

5.3. Oncotherapy-induced changes

Until 2005, treatment of GBM was limited to surgical resection and subsequent radiotherapy. Thanks to technical advances the standard whole brain radiotherapy was later replaced by conformal brain irradiation. Additionally, several trials were made with dose escalation, stereotactic radiosurgery and brachytherapy but none of them proved to be effective, so the present approved protocol contains 2x30=60 Gy

conformal irradiation. Later, therapeutic strategies expanded to include chemotherapeutic agents, which were used alone or combined with irradiation. In the era " before temozolomide", several drugs were used to treat GBM—platinoids, taxanes, topoisomerase inhibitors and other alkylating agents—but since 2005 chemoradiation with temozolomide has been considered as gold standard treatment.

Since the present therapy of GBM is not effective enough there is a need for the reveal of gene expression and protein changes. These shifts act upon cell motility, cell membrane content and the composition of ECM which have an effect on glioblastoma cell invasion and thereby infulence the effectiveness of the therapy. The deeper knowledge of these alterations can discover the limits of the present therapeutic protocol and can open the way for setting new investigational targets. Today we have little knowledge about the detailed mechanisms of the concurrent treatment, the effects of the therapy on the infiltration is also unclear and studies by now have used cell cultures, not human brain tissues. In our experiment we analyzed the effect of concurrent chemoradiation (containing temozolomide and conformal radiotherapy) on tumor invasion: only the RNA level of MMP-9 and the protein level of brevican showed significant decrease after oncotherapy. None of the other molecules showed significant change.

Since MMP-9 is a matrix-degrading enzyme, its level in glioblastoma tends to increase compared to nontumor tissue. Trog D. et al. examined the effect of temozolomide and irradiation on GBM cell lines, and they found a significant increase of metalloproteinase in the surviving cells, which correlated with GBM's aggressive

and infiltrative nature. In our human samples, the mRNA level of MMP-9 decreased remarkably after oncotherapy; however, much lower expressional decrease was detected in protein concentration. This represents the major effect of antitumor agents on DNA replication, which has only a minor impact at the protein level. Nakada M. et al. and Held-Feindt J. et al. found a clear correlation between brevican expression and the activity of cell invasion. In our study, the significantly decreased level of brevican due to oncotherapy indicates that concomitant chemoirradiation has no measurable effect on tumor invasion.

Based on our results it can be assumed that concurrent chemoradiation does not have a significant effect on the invasion of glioblastoma cells and as a result of the treatment a more aggressive subpopulation is probably selected out with a greater invasion potential. Considering this, our research contributes to the understanding of the fundamental fiasco of GBM treatment and on the other hand supports the need for developing new, targeted anti-invasional treatments.

6. SUMMARY

Glioblastoma multiforme is the most common adult primary brain tumor and has one of worst prognostic features among all diseases. One of the reasons of the inevitable recurrence is its highly infiltrative feature. Tumor cells invading the surrounding tissue induce changes in the expression of the peritumoral extracellular matrix

components. Concurrent chemoradiation as the gold standard treatment of glioblastoma improved overall and progression-free survival with months indeed, though it does reach a real success. One of the possible causes concerning the ineffectivity of the temozolomide based chemotherapy is that the treatment is basicly anti-proliferative and does not affect the infiltration of the tumor cells. Peritumoral tissue changes and possible alterations in the invasivity due to oncotherapy can be detected at molecular level through shifts in the expression levels of the inratumoral and peritumoral ECM components. Focusing on that, we determined the mRNA and protein levels of twenty invasion related molecules. QPCR was used for measuring mRNA and quantitative proteomic analysis was performed for protein level detection. The molecules were measured in tumor-free brain tissue (9 samples), peritumoral tissue of glioblastoma (9 samples) and intracerebral adenocarcinoma metastasis (9 samples) and in glioblastoma before (15 samples) and after (16 samples) concurrent chemoradiation.

Establishing the invasion pattern of the studied tissues our results highlight some ECM molecules that play a possible role in averting the invasion of metastatic cells and the incapacity of preventing glioblastoma cell migration: CD168, fibronectin, MMP-9, tenascin-C and tenascin-R. In addition it can be assumed that the chemoradiation does not have any considerable effect on the composition of glioblastoma ECM and hereby inefficent regarding the infiltrative nature of the tumor.

According to our opinion the matching of anti-proliferative products with anti-invasive agents in the treatment of glioblastoma is worth consideration.

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Candidate: Gábor Hutóczki
Neptun ID: G5FXRG
Doctoral School: Doctoral School of Neurosciences

List of publications related to the dissertation

1. Klekner, Á., **Hutóczki, G.**, Virga, J., Reményi-Puskár, J., Tóth, J., Scholtz, B., Csősz, É., Kalló, G., Steiner, L., Hortobágyi, T., Bognár, L.: Expression pattern of invasion-related molecules in the peritumoral brain.
Clin. Neurol. Neurosurg. 139, 138-143, 2015.
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Total IF of journals (publications related to the dissertation): 2,982

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