Chapter 1

Brain Tumor Invasion and Angiogenesis

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Additional information is available at the end of the chapter

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1. Introduction

It is a well-known fact that effectiveness of oncotherapy in brain tumors remains under the expectations in comparison to anaplastic tumors of other organs. Knowing the very modest survival rates enormous efforts of neuro-oncological researches has been made, but only partial success is produced. Beside the extremely high proliferation rate of high grade glioma cells researches established the highly intensive invasiveness and angiogenesis as the main reasons of treatment failure. In this chapter the main molecular mechanisms of brain tumor invasion and angiogenesis will be discussed followed by the hopeful treatment possibilities that are already in studies and will be achievable in the near-future.

2. General a spects of glioma invasion

Malignant gliomas are the most common primary brain tumors. They are associated with the shortest survival time explained by their early recurrence due to their deep invasion of the normal brain, which makes them practically impossible to remove completely. Invasive anaplastic gliomas are almost invariably fatal, recurring close to the resection margin in almost all cases. Interestingly, primary brain tumors have a strong tendency to invade their environment, but with rare exceptions, do not metastasize outside the brain. [1-3].

To understand the invasion behaviour of gliomas, the cellular and molecular events of peritumoral infiltration have to be discussed. The most important medium for this process is the extracellular matrix (ECM). The ECM comprises a considerable proportion of the normal brain volume. The extracellular space (ECS) of the healthy brain tissue volume is approximatley 20%. The extracellular volume fraction in the majority of primary brain tumors is significantly increased, representing about 48% of the total tumor tissue volume especially in

high grade gliomas. The structure and compounds of the ECM of the brain tissue have many specific differences from other human organs. The ECM of the brain contains mainly macromolecules like glycosaminoglycans (GAGs) and proteoglycans (PGs), and only moderately express fibrillary glycoproteins (e.g. collagens, fibronectin, elastin or reticulin). The compounds of ECM glycoproteins play a crucial role in peritumoral invasion forming structural elements for cellular attachment and migration. There is much evidence that ECM components can modulate brain tumor growth, proliferation, and invasion by many different mechanisms. Thus extracellular matrix plays a pivotal role in the tumorous infiltration of the surrounding tissue. The presence and functions of hyaluronic acid (hyaluronan, HA), PGs and various types of GAGs have already been intensively investigated to clarify the molecular mechanisms of invasion, and a positive correlation has been established many times. To allow cell adhesion and migration, the ECM components interact with specific receptors on the cell membrane, such as integrins, CD44, or CD168. Some proteases and synthases also strongly influence invasiveness because of their capacity to alter the actual levels of the ECM molecules or to degrade the pericellular network. [4-16]

Using the ECM macromolecules to their active movement, glioma cells infiltrate the enviroment and form it similar to the tumor tissue. The process of the peritumoral invasion depends on the confrontation zone of the tumor cells and the non-neoplastic cells and ECM. Glioma cells express mainly adhesion receptors and proteases, while host cells produce macromolecules to maintain original structure and to inhibit invading cell movement. Since brain ECM has no strong fibrillar, collagen-rich network, the brain parenchyma remains soft, that can not hinder significantly the migration of tumor cells.

In case of glial cell tumors there are two main factors that significantly promote peritumoral infiltration. First is the normal structure of the brain parenchyma composed mainly by tracts in the white matter and basement membranes, which are suitable for guiding cell migration. Second is the increased ability of glia cells to migration. Both factors are special for the brain and they can be easily understood knowing the connection of development, structure and function. [17, 18]

From neuro-oncological point of view the increased glioma cell mobility and extensive peritumoral infiltration leads to the following problems:

a. A. Total extirpation of a low grade tumor is not an easy and evident technical tool of therapy. This is one main reason why these tumors are “semi-benign” tumors. Thus in spite of the macroscopically radical surgical removal, the recurrence rate of these tumors is very high, and full recovery is not a general event.

b. B. In case of high grade tumors, neither open surgery, nor stereotactic radiosurgery can achieve radical tumor removal. This experience can explain the local recurrence that appears in almost every case.

c. C. Local chemotherapeutical treatment (intraparenchymal or post operatively administered intracavital drug) has low effectiveness.

3. Molecular aspects of glioma invasion

Molecules that are responsible for the cell migration are divided in three groups:

1. Cell-membrane associated molecules (receptors and adhesion molecules).
2. Extracellular matrix (ECM) components (targets for the receptors).
3. Enzymes that are synthesizing or lysating the ECM components.

3.1. Cell-membrane associated molecules (receptors and adhesion molecules)

Molecules with evident role in peritumoral invasion are located either on the cell surface, or form transmembrane structure. The main representatives of this group are the receptors and adhesion molecules as detailed below.

The **Ig superfamily** contains molecules in the cell membrane consisted of immunoglobulin-like and fibronectin type III domains involved in cell–cell adhesion. The superfamily includes the integrins, a variety of cell adhesion molecules (CAMs) with distinct ligand-binding specificities, namely ICAM (intercellular), NCAM (neural), Ep-CAM (epithelial), L1-CAM, VCAM (vascular), ALCAM (activated leukocyte), and JAM (junctional adhesion molecule), among others. [19]

The **integrins** are the most common molecules that serve for glioma cells to adhere to ECM. These molecules are heterodimeric transmembrane glycoproteins consisting of non-covalently linked α and β chains, which both determine ligand binding strength and specificity. Eight distinct α and 18 β chains combine to form about 24 different heterodimers. They can interact with two groups of ligands: some of the ECM proteins, such as fibrinogen, fibronectin, vitronectin, and cell surface molecules, that are members of the immunoglobulin superfamily. Regarding the many different heterodimers, each cell type maintains a specific and activation-dependent integrin repertoire and consequence ligand preference. The cytoplasmic integrin domains connect to signalling proteins and to the actin-cytoskeleton mediating intracellular signal transduction and cell movement. This function definietly demonstrate the dynamics of cell–ECM interaction as cells move along a substrate. Thus, integrins are prominently important mediators for cell adhesion and migration. They also interact with growth hormone receptors and contribute to cell–cell contacts due to direct interactions with counterpart cell receptors. On the other hand, focal contacts mainly depend on the ECM-compartment and on the cell type. Different integrins are known to be involved in that process. Integrin $\alpha 5\beta 1$ binds to fibronectin, $\alpha 6\beta 1$ or $\beta 4$ binds to laminin, $\alpha v\beta 3$ binds to fibronectin, vitronectin and tenascin-C and $\alpha 2\beta 1$ binds to fibrillar collagen. Some of the integrins are directly connected to malignant behavior of gliomas. Neutralizing antibodies to $\beta 1$- and $\alpha v\beta 5$-integrin lead to decreased glioma migration in vitro. It was also demonstrated, that tenascin increases in vitro motility of human gliomas through interaction with $\beta 1$-integrins. Inhibition of $\beta 1$-integrins leads also to decreased motility, whereas inhibition of αv-integrin causes increased motility. The integrin $\alpha v\beta 3$ plays a central role in glioma invasion. Increased expression of integrin $\alpha v\beta 3$ results in increased motility of glioma cells with a de-

crease in apoptosis sensitivity. Furthermore, inhibition of integrin αvβ3 decreases glioma cell motility. Integrins αvβ3 and αvβ6 interacting with tenascin was proved to mediate adhesion rather than migration. Expression of β5-integrin is correlated with in vitro invasiveness and migration of human glioma cells. However α-actin expression and linkage of integrins to the cytoskeleton is related to glioma aggressiveness and poor prognosis in WHO II and III astrocytoma. [20-33]

Integrins mediate also activation of **focal adhesion kinase (FAK) that** associates with β1- and β3-integrins, which can trigger FAK phosphorylation. It is a non receptor tyrosine kinase overexpressed in invasive glioma cells, and its expression correlates with tumor recurrence and invasiveness in many tumor types. FAK is activated either by integrin mediated adhesion to ECM or by growth factor stimulation and it induces cell migration. Induction of FAK can protect cells from apoptosis. [34-41]

The **neural cell adhesion molecule (NCAM)** is expressed mainly by developing neurons. It is downregulated during embryogenesis and re-expressed again once differentiation is initiated. Overexpression of NCAM decreases glioma cell motility in vitro. In drug-resistant glioma cell lines NCAM expression is reduced and integrin-expression is increased that help to explain decreased chemosensitivity in invading glioma. [42- 45]

CD44 is the most important HA-receptor expressed by every nucleated cells in vertebrates. CD44 is a transmembrane glycoprotein belonging to the immunoglobulin receptor superfamily. Besides the standard form (CD44s), multiple splice variants encoded by variable exons v1–10 (CD44v1–10) can be identified depending on the cell differentiation and activation state. Interactions of CD44 with numerous other molecules, such as collagens, laminins and fibronectin, have been proved in vitro. CD44 is consisted of four functional domains: amino terminal domain, stem structure, transmembrane domain and cytoplasmic domain. The amino terminal domain can link to the ECM components such as HA and other GAGs. The stem structure domain binds the amino-terminal domain and transmembrane domain. The transmembrane region is probable responsible for the association of CD44 with lipid rafts. The cytoplasmic domain of CD44 is connected to the cytoskeleton via ankyrin and other proteins that is necessary to cell adhesion and motiliy. CD44 can be cleaved to two parts, and both the extracellular and intracellular components of CD44 promote cell migration. CD44 also interacts with various regulatory mediators to cell signaling pathways. Through these connections CD44 promotes MMP-mediated matrix degradation, tumor cell growth, migration and invasion and its expression correlates well with invasion potential of glioblastoma. [46-54]

The **receptor for hyaluronate-mediated motility** (**RHAMM**) is also a HA-binding protein expressed on the cell surface and also in the cytoplasm, cytoskeleton and nucleus. Interaction of HA with RHAMM induces many cellular signaling pathways in connection to protein kinase-C, FAK, MAP kinases, NFκB, RAS, phosphatidylinositol kinase (PI3K), tyrosine kinases and cytoskeletal components. CD44 and RHAMM probably have redundant or overlapping functions, but it is evident that interactions of HA with CD44 and RHAMM are necessary for tumorigenesis and tumor progression. [55-58]

Syndecans are a family of transmembrane heparan sulphate proteoglycans with four members, syndecans 1 to 4. Syndecans are co-receptors by binding their ECM ligands in conjunction with other receptors, mainly integrins. Through their heparan sulphate side chains, syndecans may further take part in other ligand binding, like VEGF, fibronectin and antithrombin-1. Linking syndecan to fibronectin is modulated by tenascin-C. Syndecan-1, -3, and syndecan -2, -4 bild two different structural subgroups. Syndecan-1 is expressed generally in fibroblasts and epithelial cells (especially in keratinocytes), but normally there is only a moderate presence in endothel and neural cells. Syndecan-3 dominates in neural cells, but not in epithelial cells, and syndecan-4 can be found mainly in epithel cells and fibroblasts, while it is poorly expressed by endothel and neural cells. Syndecans have four main function: 1. activation of growth hormon receptors; 2. cell adhesion to ECM components such as collagens type I, III, V, fibronectin, thrombospondin and tenascin; 3. cell-to-cell adhesion (e.g. syndecan-4 and integrin linkage takes part in intercellular interactions; 4. tumor suppression (anti-invasive effect by keeping tumor cells together) or tumor progression (depending on tumor histology and growth phase). [59-62]

Cadherin superfamily is also an important group of adhesion molecules regarding glioma invasion. Cadherins are transmembrane proteins compound of several tandemly repeated cadherin domains that interact in calcium-dependent homophilic cell–cell contacts. The cadherin superfamily consists of more than 100 different members, with E- (epithelial) and N- (neural) and P-cadherin, most intensively expressed in epithelial and neural tissues, respectively. Desmosomal cadherins (desmoglein and desmocollin) provide a linkage to the intermediate filament network through connection with cytosolic proteins (desmoplakin, plakoglobin and plakophilin). Adherens junctions play a pivotal role in embryonic development as well as in the maintenance of tissue architecture in adults. Cadherins are linked to the actin-cytoskeleton network through catenins (α-, β-catenin, plakoglobin and p120ctn), thereby providing molecular lines of communication to other cell–cell junctions and to cell-substratum junctions. Cadherin cluster forms a transmembrane core of adherens junctions at sites of the cell–cell contacts. During tumor progression decreased cadherin function is correlated with de-differentiation, metastasis and poor prognosis. In glioblastoma N-cadherin cleavage is regulated by ADAM-10 that promotes tumor cell migration. Furthermore, aberrantly processed proN-cadherin promotes cell migration and invasion in vitro, and in human glioma the level of proN-cadherin is elevated that directly correlates with the invasion potential. [63-68]

Dystroglycan is a transmembrane glycoprotein expressed mainly in sceletal muscle cells, but it can be also found in brain tissue as well. Its main function is to creat contact between the ECM macromolecules and the intracellular cytosceleton. It is linked intracellularly to dystrophin, a protein coded on the X-chromosome (lack of dystrophin causes the herediter muscle disease named dystrophia musculorum Duchenne). Dystroglycan is a heterodimeric complex consisting of non-covalently associated α and β subunits. The α-subunit connects α2-laminin, agrin and perlecan (components of the lamina basalis), the β-subunit is the transmembrane part that binds to dystrophin. Overexpression of dystroglycan decreased

the growth rate of glioma cell lines so it was found to be involved in the progression of primary brain tumors. [69-71]

3.2. Extracellular Matrix (ECM) components (targets for the receptors)

Various components of brain ECM, like GAGs and PGs are overexpressed in gliomas. These molecules are binding sites for tumor cell receptors or they can inhibit cell migration, so they take an important part in peritumoral glioma invasion, and consequently could also serve as targets for anti-tumor therapy.

Proteoglycans (PGs) are composed of a protein core and **glycosaminoglycan side chains (GAGs)**. GAGs are carbohydrate polymers containing N-acethylglucosamine or N-acethylgalactosamine and uronic acid (glycuronacid or iduronacid).

Depending on the GAG side chains the main types of PGs are chondroitin-sulphate (glycuronacid and N-acethylgalactosamine polymer and protein core), dermatan-sulphates (former name chondroitin-sulphate-B, composed of iduronacid and N-acethylgalactosamine polymer and protein core), heparansulphate (glycuronacid and N-sulphoglucosamine polymer and protein core) and keratansulphate (galactose and N-acethylgalactosamine polymer and protein core). Hyaluronic acid (hyaluronan, HA) is consisted of only GAGs (glycuronacid and N-acethylglucosamin polymer) that has no covalent bind to a protein, so it is not a PG by definition, but due to its tight relation to the PGs in general it is discussed together with them.

One of the most frequent adhesion glycoprotein in the ECM is **fibronectin**. It has a pivotal role in cell attachment, migration, differentiation and proliferation. Although its protein fragment is coded by only one gene, more isoform exits due to alternative splicing. The main cell surface receptors for fibronectin are the integrins, but it can also bind collagens, fibrin and heparan-sulphates. It is structured of two different subunits linked by disulphid bridges to each other. Fibronectin appears in two different forms: the solubile molecule can be found in the plasma, produced by hepatocytes, it accumulates at wessel wall damage and has an evident role in clot-building. The insolubile form of fibronectin is expressed by fibroblasts and mainly localized in the intercellular ECM. In tumor stroma production of fibronectin is reduced and its degradation is increased. Paralel to these changes on tumor cell surface, the expression of the fibronectin receptor $\alpha 5\beta 1$ integrin is also decreased. ECM components such as fibronectin and collagen type IV are mostly produced by the host tissue and are associated dominantly with the vessel walls in gliomas. Fibronectin is mainly degraded by MMP-2 that is specifically active in gliomas explaining partly the moderate presence of extracellular fibronectin in glioma ECM. [72-74]

Another common component of the ECM is the molecular family of **laminins**. This glycoprotein has many variants, and it is the main component of lamina basalis. It is thought to take part in cell differentiation, adhesion, migration and cell survival. Each molecule of laminin consits three different chains (α, β, and γ chain) which has 5, 4 and 3 genetic variants, respectively. Recently at least fifteen different chain-combinations have been detected in human tissues. In the lamina basalis laminins promote cell-to-cell linkage, and it forms a spe-

cific network with connection to enactin, fibronectin and perlecan. These molecules can also bind to cell surface receptors such as integrins or dystroglycans, etc. Laminins regulate glioma cell adhesion to ECM proteins in specific manner leading to cell proliferation or cell migration and up-regulation of laminin is associated with the invasiveness activity. [75-77]

Agrin is also an ECM forming PG with the capability to collect acetylcholin receptors. Normally it is indispensible in developing neuromuscular junctions during embryogenesis. Agrin is secretaed at the end of the moto-neurons, and it is also a main component of membrana basalis in various human organs taking part in cell-ECM interactions. Together with neurocan, tenascin-C and versican it is responsible for the peritumoral infiltration of gliomas. [78]

Hyaluronan(HA) is a non-sulphated, linear, high-molecularweight GAG. It differs evidently of other GAGs, because of its extremely large molecular weight (103–104 kDa) composed of 10,000 or more disaccharide repeating units, the lack of sulfate groups or epimerized uronic acid residues and because HA is synthesized at the inner face of the plasma membrane as a free linear polymer without any protein core. It has a significant waterbinding capacity, so it controls the water content of the brain interstitium. HA comprises a substantial fraction of brain ECM and is involved in many physiological and pathological processes. In normal ECM, HA sustain tissue homeostasis, biomechanical integrity, structure and some kind of tissue cohesion. In malignant tumor tissues, HA transmit signals into cytoplasm and induces cell proliferation, motility and invasion. HA binds tenascins, lecticans, the cell surface receptors including CD44, RHAMM or ICAM-1, which together contribute to ECM organization and cell–matrix interaction. Through elevating the level of MMP-9 HA also promotes peritumoral invasion by activating the protease system. Glial tumors have increased amounts of HA which facilitates invasion activity of glioma cells. [79-84]

Lecticans comprise also a family of chondroitin sulphate proteoglycans with four members (brevican, versican, neurocan, and aggrecan), whereby brevican and neurocan are brain-specific molecules. Lecticans contain HA and tenascin binding sites and thus mediates linkage in protein–PG-GAG networks. [85-86]

Brain enriched hyaluronan binding (BEHAB) molecule,also known as **brevican**, a brain-specific chondroitin sulfate PG shows dramatic upregulation in gliomas and it is also induced during periods of increased glial cell motility in development and following brain injury. Gliomas express unique brevican isoforms and the processing of this specific isoform is important for its proinvasive role. In experimentally induced tumors brevican accumulates at the invasive borders and it associates with high infiltrative profiles. Furthermore, brevican up-regulation correlates well with short survival periods of patients with high grade gliomas. Brevican expression in gliomas is restricted to membrane localization, and its presence in high-grade gliomas suggests that it plays a significant role in glioma progression. Brevican promotes activation of epidermal growth factor receptor (EGFR), increases the synthesis of cell adhesion molecules and facilitate fibronectin microfibrill presence on the cell membrane. The effect of brevican on glioma cells motility is mediated not only via EGFR signaling but also by fibronectin-dependent adhesion, and increased expression of CAMs. This motogenic signals could not be worked in the normal neural ECM, where fibro-

nectin is almost absent but it is effective in the microenvironment of glioma cells, which co-express large amounts of brevican and fibronectin in vivo. This interaction explains the distinct ability of these tumors spreading in the central nervous system. Overexpression of brain-specific isoforms of brevican proved to be correlated with ability to peritumoral invasion of gliomas. [73, 87-89]

Neurocan is a large brain specific chondroitin-sulphate PG that interacts with heparan-sulphate proteoglycan (HSPG) molecules, such as syndecan-3 and glypican-1. It has influence on cell adhesion and migration. Neurocan has two HSPG-binding domain with different affinity. In cell culture neurit outgrowth is increased by C-terminal part of neurocan. HSPGs serve also as cell-surface receptors for neurocan, and connection of neurocan to the HSPGs is necessary for the neurit growth. It was found on clinical samples that higher expression of neurocan is associated with the invasive activity of astrocytomas. [89-90]

The ECM glycoprotein **tenascin**, which forms a hexabrachion structure, can be detected in both the ECM and the perivascular tissues of high-grade gliomas. Tenascin R, a brain-specific member of the tenascin family comprising also tenascin C, X, and W, is a homotrimer with both lectican and integrin binding sites forming an adhesion link between the ECM and cells. The developed brain does not contains tenascin, but in normal brain tissue distinguishable deposits of this glycoprotein can be found in the glia limitans externa, and some tenascin was also detected in the ECS of white matter. Theres is a positive correlation between tenascin production and the malignancy or angiogenesis of astrocytomas and there is a prognostic utility of its immunohistochemical detection in ependymomas. The accumulation of tenascin in the ECS in high grade glial tumors can be one of the major factors leading to the critical increase in ECS tortuosity and the simultaneous enlargement of the ECS. It has been arised that the ECM distribution is modified at the brain-tumor zone of confrontation and the presence of tenascin in this zone represents a negative prognostic factor in pediatric ependymomas. Tenascin-C is overexpressed in both low and high grade astrocytomas as well. In cultured brain-tissue tenascin-C is produced by the endothelial cells. It takes an important part in various cellular mechanisms like heamagglutination, T-cell immunsuppression, angiogenesis, chondrogenesis and it also has some antiadhesive effect. Tenascin subunits contain EGF- and fibronectin-like repeated sequences that are responsible for the growth inducing effect. Tenascin-C enhances migration of endothelial cells and phosphorylation of focal adhesion kinase (FAK). Tenascin-C signaling is mainly mediated by integrin-β1 which interacts with FAK. Tenascin-C is produced by the glioma cells rather than by the invaded brain and it improves aggressive behaviour and invasion activity of grade II astrocytoma cells in vitro and in vivo. Furthermore, expression of tenascin-C can be used as prognostic factor in grade II astrocytomas showing correlation with ability of tumor recurrence. Beside this, low tenascin-C expression was found to be associated with prolonged average survival time in glioblastomas and highest tenascin-C expression could be detected at the border of the malignant gliomas. [91-104]

Versican (also known as VCAN or CSPG2), a chondroitin sulfate PG, is one of the main components of the ECM, expressed almost in all human tissues. Versican takes part in normal tissue development, but its increased expression can be also detected in most malignan-

cies. Elevated versican production occurs in either the tumor cells or the stromal cells surrounding the tumor. Increased versican expression strongly correlates with poor outcomes for many different tumor types. Versican regulates a wide variety of intracellular processes including cell adhesion, proliferation, apoptosis, migration and invasion via the chondroitin and dermatan sulfate side chains. In addition, the versican G1 and G3 domains can interact with various intracellular or extracellular molecules. In addition to HA, versican associates with tenascin-R, fibulin-1 and -2, fibrillin-1, fibronectin, P- and L-selectin, and many chemokines. It also binds to cell surface proteins including epidermal growth factor receptor (EGFR), CD44, and integrin β1.

A number of proteinase families are capable of generating the proteolytic fragments of versican. Matrix metalloproteinase (MMP)-1, -2, -3, -7, and -9, ADAMTS-1, -4, -5 and -9 cleave versican and generates proteolytic fragments.The accumulation of proteolytic fragments of versican play an important role in cancer progression. The regulation of G1 and G3 versican levels by proteases is known to be important in regulating cancer cell motility and metastasis. Through the EGF-like motifs in the G3 domain versican can stimulate cell proliferation and its G1 domain destabilizes cell adhesion and promotes cell growth. Versican expression is associated with a high rate of proliferation and it is localized in HA-rich tissues and also accumulated in perivascular elastic tissues involved in peritumoral invasion. These features of versican make it a proliferative, anti-adhesive and pro-migratory molecule that facilitates tumor cell motility. In clinical samples the association of versican to invasiveness of astrocytoma could be evidently demonstrated. On the other side, the decreased expression of versican V0 and V1 isoforms in glioma ECM can be related to the marked local invasivity and rarity of extracranial metastasis of gliomas. [105-111]

3.3. Enzymes that are synthesizing or lysating the ECM components

Matrix metalloproteinases (MMPs) are the most common proteases that degrade ECM to create the space for invading glioma cells**.** MMPs belong to the zinc-dependent endopeptidase together with adamalysins, serralysins and astacins. MMPs take part in remodelling after tissue damage, cell migration, differentiation and angiogenesis. At least 28 different types of MMPs are identified composing a protease family that is able to degrade practically every component of the ECM. Due to their function, MMPs also play evident role of activating mechanism by cleavage metabolits of inactive molecules. MMPs are overexpressed in glioma cells compared with normal brain tissue. MMP-2, MMP-3 and MMP-9 activity correlates well with glioma cell migration and invasion. [46, 112, 113]

Cathepsin-B is a cystein protease involved in protein degradation primarily within intracellular lysosomes but it takes evidently part in degradation of ECM-proteins. In order to be able to interact with ECM proteins, the lysosomal enzyme is secreted from its intracellular localization. Thus cathepsin B appears on the surface of glioma cells, where the enzyme can interact with the surrounding matrix components. Cathepsin-B is overexpressed in gliomas. Downregulation of cathepsin B in human glioma cells leads to decreased invasiveness in matrigel-assay and coculture experiments. Furthermore, downregulation of cathepsin-L in

human glioma cells correlates with decreased invasiveness and increased sensitivity to apoptotic stimuli. [114-118]

4. Invasion process of tumor cells

Knowing the invasion potential of primary brain tumors, many of the molecular mechanisms of peritumoral infiltration have been already studied and some of the invasion processes have been defined. During malignant transformation, invasiveness is determined by the complex functions of tumor cells of distinct histological types. A four-step model of invasion has been applied, that is also valid for brain tumors. This model contains the following steps: 1) the tumor cells at the invasive site detach from the growing primary tumor mass; 2) they adhere to the extracellular matrix (ECM) via specific recebptors; 3) proteases secreted by the glioma cells locally degrade the ECM components, forming a pathway migration into the surrounding tissue, and 4) tumor cell movement due to cytosceletal processes. Each step of the peritumoral invasion requires a harmonized cooperation of numerous molecules resulted in active cellular movement into the normal brain parenchyma. [119, 120]

The detachment of invading glioma cells from the primary tumor mass is a complex process comprising the following steps: 1) Destabilization and disorganization of the cadherin mediated junctions that hold the primary mass together. 2) Decrease expression of cell adhesion molecule which provides adhesion to the primary tumor mass. This leads to a reduction in gap junction formation. Cell–cell communication is necessary for growth control and differentiation, and it is mainly achieved through gap junctions. Increased malignancy of gliomas is accociated with reduced in situ gap junction formation, and invasion of gliomas. 3) Cleavage of CD44, which anchors the primary mass to the ECM. This process is mediated by metalloproteinase ADAM. [119-123]

Tumor cell adherence to the ECM components is mediated by specific cell surface or transmembrane receptors like integrins binding to laminins, fibronectins and collagens or CD44 to hyaluronan.

Degradation of ECM components occurs due to the local enhancement and activation of protease suc as MMPs, hyaluronidase, cathepsins and chondroitin suphatase.

Due to migration the glioma cell must interact with the surrounding ECM, which forms a mechanical barrier to the cells, and serves as a substratum for traction for the moving cells. For cell movement changes in cell morphology occur: the cell becomes polarized and membrane protrusions develop, including the extensions at the leading edge of pseudopodia, lamellipodia, filopodia, and invadopodia. These extensions contain filamentous actin and various structural and signaling molecules. The formation of membrane anchors needs cytoskeletal contraction, which finally results a cell forward displacement. Glioma cell motility and contractility also require A and B isoforms of myosin II. Myosin II is the major source of cytoplasmic contractile force. Myosin II allows glioma cells to squeeze through pores smaller than their nuclear diameter, which is especially important for gliomas because the human

brain tissue has particularly narrow extracellular spaces. The connection of ECM macromolecules and cytosceleton is mediated by dystroglycans. [69, 124]

5. The possible agents for antiinvasive therapy

Tumor cell invasion into the surrounding brain tissue is mainly responsible for the failure of radical surgical resection and successful treatment, with tumor recurrence as microdisseminated disease. ECM related molecules and their receptors predominantly participate in the invasion process, including the cell adhesion to the surrounding microenvironment and cell migration. Determination of the key molecules of invasion process can help toprovide possible targets for antiinvasive therapy. Regarding peritumoral infiltration activity of glioma cells, the following molecules are supposed to serve as antitumor agents.

Cilengitide is a cyclic peptide targeting the RGD-motif of integrins blocking $\alpha v\beta 3$- and $\alpha v\beta 5$-integrin mediated interaction between endothelial cells and ECM. By targeting these integrins cilengitide could inhibit both glioma invasion and angiogenesis. Cilengitide causes significant regression of glioma xenograftsand induces apoptosis in U87 glioma cells cultured on tenascin and vitronectin. In clinical trials targeting glioma invasion, in a randomized phase II study cilengitide proved to be safe and was associated with a median survival of 10 months in recurrent glioma patients. The North American Brain Tumor Consortium (NABTC) study aimed to determine cilengitide penetration rate into GBM in human patients. This study confirmed that cilengitide is effectively delivered into primary human GBM tumors with good retention. The effect of combination therapy, such as cilengitide with XRT or with another chemotherapeutic agent, is likely to be cumulative. [125-129]

Knowing the evident role of versican proteolytic fragments in cancer progression, its possible role as target for anti-cancer therapy has been arised. Although there are only a few results regarding anti-versican therapy in glioma patients, some possible agents are notable to mention for their potential future role.The tyrosine kinase inhibitor **genistein** has been shown to block versican expression in malignant mesothelioma cell lines and in vascular smooth muscle cells. Versican G3 fragments facilitate cancer cell growth, invasion and metastasis through EGFR signaling. The selective EGF receptor inhibitor, **AG1478** prevents G3 fragment enhanced cell growth, migration, invasion and chemical resistance in vitro. **Galardin,** an antibody against the ADAMTS specific versican cleavage site inhibits glioma cell migration. **GM6001**, a MMP and ADAMT proteases inhibitor, also decreases cancer cell invasion and metastasis in several kinds of carcinoma. Other protease inhibitors such as **catechin gallate esters**, present in natural sources (green tea) selectively inhibit ADAMTS-1, -4 and -5 catabolism. [130-137]

Tumor formation of the pericellular matrix with HA and versican can be inhibited by treatment with **HA oligomers,** which can block the interaction between HA and versican, serving as inhibitors of cancer dissemination. Furthermore, disruption of the HA CD44 interaction with HA oligomers could also inhibit the growth of B16F16 melanoma cells, Therefore the application of HA oligomers can be an effective agent for inhibiting the for-

mation of vesicant-HA-CD44 complexes, providing valuable targets against tumor progression. [138-140]

Emodin (3-methyl-1,6,8-trihydroxyanthraquinone) has evident anti-invasive effect on HA-induced glioma invasion. In glioma cells emodin inhibits the TGFbeta and FGF-2 induced expression of syndecan-1. It decreses the expression of MMP-2 and MMP-9 at both transcriptional and translational levels suggesting that emodin can be a clinically valuable anticancer agent against glioma invasion. [141, 142]

Since increased MMP levels are associated with glioma invasion and angiogenesis, **marimastat**, an orally active drug that can reduce MMP levels in patients with gliomas could inhibit growing of tumor. A phase II study evaluated marimastat combined with temozolomide (TMZ) in patients with recurrent malignant glioma and good outcome was documented, but joint and tendon pain was reported in 47% of patients. [143, 144]

6. General aspects of angiogenesis

Rapidly growing tumors need to develop their own vasculature. The hypervascularisation of high grade gliomas can be visualized well on radioimaging and it can be a preoperative characteristic of glioblastoma. Furthermore, glioma angiogenesis is necessary for tumor expansion and survival, so its inhibition could be a potential tool in anti-tumor therapy.

There are two main angiogenic and invasive glioma phenotypes. Clusters of glioma cells perform single cell infiltrations into normal parenchyma independent of vasculature. Another group of glioma cells can be found around newly developed vessels in the normal brain parenchyma near to the tumor margin. These two different angiogenic and invasive phenotypes are called angiogenesis-dependent and angiogenesis independent invasions. High grade astrocytomas content both invasion phenotypes in a mixture of subclones present in different intratumoral regions. Molecular mechanisms of single cell migration were detailed above, but the role of neo-angiogenesis forms also a very important way to glioma expansion. [145]

In expanding, highly proliferate gliomas angiogenesis is activated when the pro-angiogenic stimuli dominates over the anti-angiogenic stimuli. These stimuli are mediated by factors secreted from glioma, endothelial or microglia cells, or arise from the extracellular matrix or other environmental sources like hypoxia induced cell productions. The pro- and anti-angiogenic forces are influenced strongly by tissue hypoxia and genetic alterations. The summation of these stimulileads to the so-called “angiogenic switch” in glioma angiogenesis.The most effective activator of angiogenesis in brain tumors is hypoxia that downregulates anti-angiogenic pathways and induces many pro-angiogenic ones. A well-known pathway is the HIF-1/VEGF-A pathway, which play a significant role in endothelial cell proliferation and migration. Another pathway mediator is interleukin-8, which is produced by microglia cells as a reaction to hypoxia. It is important to mention, that genetic instability of high grade gliomas provides the way of angiogenesis independently of hypoxia (such as chronic HIF

activation via phosphoinositide 3-kinase (PI3K) or mitogenactivated protein kinase (MAPK) pathways. [146-152]

After activating the "angiogenic switch", the tumor produces new vessels. The modes of new blood vessel formation in glioma occur by one of three different methods: 1) angiogenesis; 2) vasculogenesis; or 3) arteriogenesis. Angiogenesis is the formation of new blood vessels by rerouting or remodeling existing tumor vessels, and is supposed to be the main stream of neo-angiogenesis. Vasculogenesis means de novo production of blood vessels from circulating marrow-derived endothelial progenitor cells originally as the method of vasculature development in embryonic process. Since these progenitor cells have been also identified in tumors, they role in tumor angiogenesis cannot be denied. Vasculogenesis is probably regulated by tumor-secreted stromal-derived factor 1 under the control of the hypoxia-induced transcription factor hypoxia-inducible factor 1α (HIF1α). Arteriogenesis is the third mode of arteriolar networks formation representing a moderate proportion of tumor angiogenesis. [153-156]

6.1. Neoangiogenesis

The most significant way to form new blood vessels in gliomas is neoangiogenesis. Formation of new vessels from native vessels begins with breaking down the original vessel wall. The process of blood vessel breakdown is composed of three main phases. The first event in forming new vessels from existing ones is the disintegration of the vessel wall. Angiopoietin-1 (Ang-1) and its receptor Tie-2 play a pivotal role in this phase. Normally, Ang-1 binds to Tie-2 achieving a close association between pericytes and endothelial cells that is necessary for vasculature stability. In rapidly proliferating tumors like glioblastoma, tissue hypoxia increases and it induces Ang-2 upregulation in endothelial cells whereas Ang-1 is accumulated tumor cells. Increased Ang-2 expression, which is an antagonist of Tie-2, leads to the initial regression of blood vessels. Beside these, matrix-metalloproteinase (MMP)-2 expression is induced via Tie-2 signaling, and in conjunction with VEGF promotes angiogenesis. The second phase is the breakdown of ECM to provide place for the migration of endothel cells to form new blood vessels. Following dissolution of native vessel wall, degradation of the vessel basement membrane and relating ECM is the necessary condition for endothelial cells for invasion the surrounding microenvironment. MMPs play an integral role in this phase. In case of glioma angiogenesis, the collagenases MMP-2 and MMP-9 are involved in this process and their expression correlates with a poor prognosis in gliomas. Expression of MMP-2 and MMP-9 is also induced by hypoxia and through their proteolytic activity interaction of endothelial cells and tumor-ECM contents like VEGF and fibroblast growth factor (FGF) occurs. [157-166]

The third phase to form new blood vessels is the migration of endothelial cells. After dissolution of the basement membrane of the blood vessels and decomponent ECM, endothelial cells begins to proliferate and migrate toward tumor cells that expresses pro-angiogenic factors. Due to this process cell surface adhesion and migration molecules, such as integrins and CD44 upregulates.The activated endothelial cells secrets platelet-derived growth factor (PDGF) that induces pericytes to participate in creating a new basement membrane. For this

reason beside migration of endothelial cells, pericyte migration also occurs as a necessary event of vasculogenesis. [167-169]

At the end of tumor blood vessel formation a significant change occurs in the extracellular environment, caused by increased expression of embryonic ECM molecules, such as tenascin-C. Elevation of VEGF and Ang-2 levels can be also detected, that probably explains the leakiness and pathologic structure of the new vessels. The result of glioma angiogenesis are highly tortuous dilated vessels and lots of small diameter vessels with alterations in endothelial cell adhesion molecule expression and disrupted basement membrane. [170-174]

7. Molecular aspects of glioma angiogenesis

Angiogenesis is mainly induced through growth hormone receptors, especially through the **vascular endothel growth factor receptor (VEGFR)**. This is a transmembrane receptor with an extracellular antibody-binding domain (for vascular endothel growth factor (VEGF)) and an intracellular tyrosin kinase domain stimulating the PI3K/Akt pathway. In tumor angiogenesis the effect of VEGFR can be increased either by receptor overexpression on the cell surface or by mutation of the receptor that without a hormone-ligand or by only a moderate ligand connection it keeps on a permanent stimulus.

Regarding glioma angiogenesis not only VEGFR but the hormone ligand **VEGF** has also an evident role in the process. There are more types of VEGF. Specifically, VEGF-A is upregulated in glioblastoma and it is produced by many cell types, such as tumor cells, stromal, and inflammatory cells. VEGF-A is primarily induced by tissue hypoxia and it regulates endothelial cell survival, proliferation, permeability and migration mainly through the VEGF-receptor 2 (VEGFR2). VEGF can also be derived from the tumor-ECM. Beside the increased amount of VEGF, the receptors VEGF-R1, VEGF-R2 and VEGF-R3 are upregulated on endothelial cells in glioma in comparison to normal brain. [175- 182]

Other growth factors have also influence on angiogenesis. Epidermal growth factor (EGF), **basic fibroblast growth factor (bFGF)** and **platelet derived gfrowth factor (PDGF)** facilitates VEGF expression. The result of pathologic increased VEGF signaling in tumors is immature, highly permeable blood vessels with deteriorated blood-brain-barrier (BBB) function and subsequent parenchymal edema. In glioma, bFGF is expressed by tumor cells and endothelial cells but it can be also accumulated and stored in the extracellular matrix of glioma. [183-186]

In rapidly proliferating anaplastic gliomas oxigene supply remains constantly under the necessity, thus hypoxia remains a permanent stimuli for angiogenic factors. It seems to induce not only the secretion of growth factors, but also **interleukin-8 (Il-8)**, a chemokine released by microglia, and Il-8 is expressed in adult glioma at levels correlating to tumor grade. In glioma the interleukin-8 mediated angiogenesis is regulated by the tumor suppressor protein ING4 through the transcription factor NFκB. [187-189]

Interestingly, there are some molecules involved in neuronal pattering during embryogenesis that have similar functions in vascular pattern during tumor angiogenesis. One of these molecules is the **semaphorin**, that induces signal pathway through neuropilins and plexins. Neuropilins are expressed on vascular endothelial cells and function as receptors for VEGF. Their activation leads to pro-angiogenic responses even in the absent of the classical VEGF-R2 signaling and blocking neuropilin-1 can decrease tumor angiogenesis and growth. [183, 190-192]

Beside growth factors and their receptors, there are some ECM components that are overexpressed in glioma vessels in comparison to normal brain tissue, and have some stimulating effect on angiogenesis. One of the most important ECM proteins with an evident role in angiogenesis is **tenascin-C**, which is normally not expressed in the adult brain, but in glioma it can be found at the invading tumor border in the region of angiogenesis. Tenascin-C facilitates endothelial cell migration and induces VEGF expression and focal adhesion kinase phosphorylation, which are both important for angiogenesis. Another ECM protein involved in angiogenesis is **fibronectin**. The oncofetal form of fibronectin is typically only expressed during embryogenesis, but it is also produced in GBM, and it is localized to the tumor vessels. **Laminin-8** a member of the laminin family in ECM is expressed in vascular basement membrane of GBM. Its blocking in an animal model of GBM resulted in decreased tumor microvessel density and increased survival. **Versican** is also an important ECM component of the tumor angiogenesis process. The versican G3 domain facilitates endothelial cell adhesion, proliferation, and migration in vitro and blood vessel formation in nude mouse tumors. Furthermore, G3-domain expressing cells produce increased levels of fibronectin and VEGF, suggesting their common functions in angiogenesis. [193-197]

8. The possible agents for anti-angiogenic therapy

Since VEGFR play the most significant role in tumor angiogenesis, its inhibition bears the most effective possibility for decrease tumor growth. The VEGFR is a transmembrane tumor cell receptor, so blocking antibodies could close down its effect. On the other side blocking the intracellular tyrosine-kinase domain could also inhibit the activation of the signaling pathways. The latest way came into the front in past few years, when small-molecular tyrosine kinase inhibitors proved to be effective in vitro against glioma cell lines. Beside these, blocking the VEG-factor itself can also definitely decrease the stimulating effect of the receptor.

8.1. VEGF-blocking

The most known VEGF neutralizing antibody is the bevacizumab that is already a possible tool of the oncotherapy for glioblastoma. In recurrent glioma patients treated with bevacizumab combined with the chemotherapy agent irinotecan the median survival can be prolonged. As the result of a significant antitumor effect 63% radiographic response, 6-month progression-free survival in 32% of GBM patients could be achieved. Based on these favora-

ble observations further clinical trials have been initiated to combine bevacizumab with temozolomide, the current standard of care for newly diagnosed glioblastoma patients. Another clinical trial suggests that the presence of tumor hypoxia markers predicts probable radiographic response and better survival of patients treated with combinant chemotherapy of bevacizumab and irinotecan. Gliomas treated with bevacizumab often appear as nonenhancing infiltrating laesions on MRI proving the reduced vascularity beside the ongoing invasion, so induction of anti-angiogenic therapy combined with anti-invasive therapy seems to be a possible treatment method in the future. [198-203]

8.2. VEGF-receptor blocking

Anti-angiogenic therapy with VEGF receptor inhibitor **sunitinib** normalizates tumor vasculature, so it elevates intratumoral level of temozolomide due to the improved vessel functions. **Cediranib** is a pan-VEGFR tyrosine kinase inhibitor, while **enzastaurin** is a protein kinase-C inhibitor. Both agents are already in studies. **Sorafenib** is a multikinase inhibitor, that suppresses angiogenesis by inhibiting VEGFR and PDGFR activities in endothelial cells. Sorafenib-treated mice showed significant suppression of glioblastoma cell proliferation, increased apoptosis and autophagy, and reduction of angiogenesis in vivo, phase II trials of sorafenib in patients with malignant gliomas were inducted. **Imatinib** is a kinase inhibitor of PDGFR, c-kit, and bcr-abl. In vitro studies of imatinib on glioma cell proliferation describe, that it is cytostatic agent at low concentration whereas at high concentrations it has cytotoxic effect. Imatinib monotherapy against malignant gliomas has failed to show any significant clinical benefits probable because of the moderate drug penetration across BBB and the inhibition of PDGFR alone can be insufficient to inhibit growth of malignant gliomas. In spite of these its use in combination therapy is still an interesting theme. [204-211]

8.3. Other target molecules for anti-angiogenic therapy

Tenascin-C is mainly expressed in hyperplastic vessels and it promotes migration of endothelial cells in astrocytic tumors. Therefore, blocking tenascin with an antibody to inhibit angiogenesis seems biologically reasonable, so a tenascin-specific antibody radiolabeled with I-131 was tested in patients with high-grade gliomas. The phase II studies with tenascin-blocking antibody in malignant glioma reported about a slight increase in survival time. [101, 195, 212-214]

Another ECM protein that has anti-angiogenic effect in glioma is secreted protein acidic and rich in cysteine (SPARC), also known as **osteonectin** or BM-40. Osteonectin takes part in a number of basic biologic functions, including migration, proliferation, and survival. Expression of SPARC in the nervous system is restricted normally to the angiogenic microvasculature, such as in the region of locus coeruleus and retinal astrocytes, but is not expressed in the cerebral cortex. In contrast, osteonectin is present in both tumor cells and endothelial cells in gliomas of all grades, and it is also expressed by endothelial cells and astrocytes in the adjacent tissue. Osteonectin suppresses tumor angiogenesis via inhibition of VEGF expression and secretion. [215-221]

8.4. Endogenous anti-angiogenic factors

A number of endogenous anti-angiogenic factors have been described that play pivotal role in tumor angiogenesis. Identifying these factors could offer some anti-cancer agent for neuro-oncological therapies. One of the best known endogenous anti-angiogenic proteins is **angiostatin.** It is mainly derived from degradation of plasminogen by proteases cathepsin-D and MMPs. In vivo studies in mice proved that angiostatin inhibits glioma angiogenesis and growth. The **thrombospondins (TSPs)** are another family of proteins that serves as an anti-angiogenic factor. In normal tissue TSP1 is produced by platelets, endothelial cells, and smooth muscle cells. Similar to angiostatin, **endostatin** is also an anti-angiogenic molecule created in glioblastoma basement membrane by proteolytic cleavage of collagen-18 by elastase, cathepsin-L, and specific MMPs. The endostatin-mediated signaling has more angiogenic inhibitory mechanism by binding to a5b1 integrin, inhibition of VEGF-R2, reduction of focal adhesion kinase-mediated endothelial cell migration, and suppression of pro-angiogenic MMP-2. A further factor is the angiogenesis inhibitor-1 (BAI1), also known as **vasculostatin,** that is produced only in glial cells and neurons of normal brain but not in blood vessels. Since vasculostatin is defnietly reduced in glioblastomas, its role in suppressing angiogenesis is glioma is strongly supposed. [222-231]

9. Conclusion

There are no simple and evidently succesful protocols for therapy of primary brain tumors. The intensive proliferation activity, the significant peritumoral infiltration and increased angiogenesis altogether are responsible for the extremely high recurrence rate of gliomas. The failure of recently administered chemotherapy arises the requirement of combination therapy. Thus besides searching a highly specific tumor marker, establishing the molecular spectrum of these tumors can be suggested. Supporting this theory, the mRNA expression pattern of the invasion-related molecules was found to be highly specific for various different histological tumor groups. So determination of the genetic signature of invasion of a glioma is thought to help in screening exact molecules as targets for individual chemotherapy. [89] Furthermore, complexity of oncotherapy with combination of antiproliferation, anti-invasive and antiangiogenic drugs could bring benefits in treatment effectiveness against brain tumors in the future.

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