Mechanisms of tumour cell invasion and metastasis formation in the brain: a review

by

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ABSTRACT

Primary brain tumours and metastasis in the brain are both uncontrolled diseases leading to death, no matter which aggressive surgical and adjuvant treatment is undertaken. High grade gliomas are the most common type of primary brain tumours and brain metastasis arise from any type of primary cancer in the body. Both diseases are extremely aggressive and patients die mostly from the progression and invasion of the tumour. Understanding the underlying biology of brain tumours and metastasis is crucial hoping to control their behaviour. As a first part in this review, we focus on the molecular mechanisms involved in primary brain tumours formation, especially gliomas cell invasion. This involves current understanding of the molecular mechanisms responsible for: 1) the dysregulation of proliferation, 2) increased migration, 3) recruitment of local blood vessels and involvement of angiogenic factors for the migration of cancer cells and, 4) some insight into the concept that cancer stem cells, at the origin of gliomas, are maintained and proliferate in a special perivascular niche microenvironment, which provides a constant source of invasive cells. We believe that these features represent the four main strategies used by brain tumours to invade local brain parenchyma. In the second part of the review, we highlight some mechanisms relevant for brain metastasis seeding, especially the initial formation of the "premetastatic niche". The recent discovery of "metastatic suppressor genes" acting at different level of the metastatic cascade might also open new fields to find anti-cancer drugs. In both diseases, primary brain tumours and metastases, the involvement of vessels and angiogenic factors are essential for tumour growth. Targeting molecular pathways involved in both angiogenesis and cancer cell migration or cancer stem cells proliferation could be essential for new anti-cancer therapies.

INTRODUCTION

Primary tumours arising in the central nervous system (CNS) and brain metastasis account for the most rapidly progressing and deadly cancers in humans. Nothing is more frustrating for a neurosurgeon to remove entirely a high grade glioma in a young patient when it is unfortunately well known that even with aggressive surgery, followed by chemotherapy and radiotherapy, his chances of survival at 3 years are less than 15%, with an overall median survival rate of 12 months [70].

For brain metastases, the picture is even worse: metastases exceed the number of primary brain tumours by at least four times and occur in about 25% patients with cancer. The overall median survival rate of patients with a single brain metastasis treated with surgery and whole brain radiotherapy is between 6 and 11 months [87].

Over the last 30 years, the management of high grade gliomas has improved. Initially, it was considered to be futile by neurosurgeons to remove a glioblastoma (GBM). Nowadays, it has become standard medical practice, providing that the tumour is accessible for surgery without causing neurological defects. These changes in treatment regimens have been possible since significant progress has been made with microsurgical techniques and complementary adjuvant therapies. For glioblastomas, hope has come from the use of temolozomide (TMZ), which significantly increases the median survival rate from 12 to 15 months, this for patients who received surgery and radiotherapy alone compared to those who had the same treatment combined with TMZ [156] [66].

There are many biological reasons why brain tumours and metastasis are so destructive and will re-grow even with aggressive initial treatment. Because of their intrinsic ability to invade the brain parenchyma, complete resection is never possible and the remaining cancer cells around the tumour resection site will inevitably restart to proliferate and invade the surrounding brain parenchyma, even if radiotherapy and chemotherapy have been

administrated. What are the mechanisms that brain tumours and metastasis have developed to maintain this immense invasion potential?

In this review, we will first focus on the mechanisms that primary brain tumours, especially gliomas, have developed to become a tumour and maintain this aggressive behaviour. We highlight four major aspects which collectively contribute to the invasive properties of primary brain tumours:

1) To become tumoural, many different mutations occur in tumour suppressor genes or developmental pathways. This maintains the proliferative characteristics of gliomas cells, a crucial aspect to keep the invasive properties of brain tumours.

2) Brain tumours have a distinct invasion potential. In fact cancer cells overexpress key molecular mechanisms used by normal cells or fibroblasts, to migrate and attach in a complex environment, composed essentially from the extracellular matrix (ECM). The fundamental interaction between glioma cells and the ECM through the attachment by integrins is highlighted. Gliomas cells themselves are capable of producing their own surrounding ECM, thus providing an adequate microenvironment favourable for migration at the leading invading front of the tumour.

3) Neoangiogenesis is crucial for the growth of brain tumours, and we review here some of the main angiogenic pathways, that have a direct effect on the migration or invasive properties of cancer cells via stimulation and/or chemoattractant properties. We highlight the role of the hypoxia inducible factor-1α (HIF-1α) considered to be the "master switch" that triggers the "angiogenic switch", a key feature in the growth of high grade gliomas. Moreover, we show some evidence that cancer cells use the perivascular space as a route of migration and as a favourable milieu to proliferate.

4) Cancer stem cells might be the source of cancer cells that participate in the growth of brain tumours. Recent reports suggests that these cancer stem cells localize in specific "vascular niches" where the close relation between endothelial cells and the specific perivascular microenvironment is determinant for their maintenance and growth potential.

Our second focus examines mechanisms used by brain metastases to invade the parenchyma. The initial stage of invasion is the detachment as single cells from the primum cancer of origin, vascular transport to the brain, and finally capillary wall transversal and formation of a "pre-metastatic niche". Local metastasis invasion follows almost the same rule as primary brain tumours invasion, except that the interface between the metastasis and the surrounding brain parenchyma is usually better defined. Metastases also recruit blood vessel from the subpial space, and angiogenesis is a determinant factor for their growth. An interesting molecular aspect is the identification of "metastatic suppressor genes" distinct from oncogenes, that can spontaneously suppress metastatic growth at any point of the metastatic cascade, and which gives new hope for anti-cancer therapies.

CHAPTER I: PRIMARY BRAIN TUMOURS

Characteristics of malignant brain tumours

Malignant brain tumours have been classified according the WHO classification, depending on the cell types that predominate [97]. The most malignant form of glioma is of grade IV according to the WHO, and is known as glioblastomas multiform (GBM). It can result de novo or as a malignant transformation of low grade gliomas. GBM are characterized by a diffuse infiltrating pattern with extensive dissemination, which makes complete surgical resection impossible [74]. Medulloblastomas, frequent in children, are believed to arise from the malignant transformation of progenitor cells of the external granular layer of the cerebellum [11]. Understanding the biology of brain tumour might give clues to what really is the "driving force" of their aggressive behaviour. Nowadays, the only histological classification of brain tumours has become almost out of date and genetic analysis plays an increasing part in the classification and treatment planning of brain tumours.

The origin of brain tumours and the "cancer stem cells hypothesis"

A fundamental key is to understand how malignant brain tumour start. Recently has emerged the novel concept that a small population of cells, the "cancer stem cells", could be at the origin of the tumour and maintain its growth. Stem cells and cancer cells, can share similarities especially their self-renewal capabilities. Tumours may originate from the transformation of normal stem cells, undergoing mutation in their growth regulation pathways [133]. To explain the origin of tumours, two models have been suggested: 1) the "stochastic model" predicts that all the cells in a tumour have a similar tumourigenic potential which is activated asynchronously and with a low frequency in certain cells. In contrast, in the

"hierarchical model" only a small subset of cells within the tumour, have a proliferative potential [133]. This latter hypothesis is in line with the new "cancer stem cells" hypothesis.

In the adult brain, a continuous production of neurons persists through life in two specific regions of the adult brain, the subventricular zone of the lateral ventricle and the subgranular zone of the dentate gyrus of the hippocampus [2, 3, 34, 35]. This presence of undifferentiated, mitotically active neural stem and progenitor cells, might function as a source of cells for transformations and mutations, giving possibly rise to "cancer stem cells" [181].

Multipotent neural stem cells in the subventricular zone give rise to three main cell types, in the mature CNS: neurons, oligodendrocytes and astrocytes [109]. Stem cells accomplish self-renewal by undergoing asymmetric divisions, by which a copy of the mother cell, together with a more mature progenitor is generated [166].

Identifying neural stem cells is possible due to the fact that they are expandable when placed in culture and stimulated with growth factors such as EGF and FGF-2 [35, 59, 60, 134]. Neural stem cells can be cultured as neurospheres [134], which has become the method of choice to study neural stem cells in vitro.

Compared to the identification of stem cells or neural stem cells, the detection and isolation of brain tumour stem cells is more challenging. Cells with neural stem cells characteristics have been first identified from surgical specimen of glioblastomas and medulloblastomas [80, 152] and express specific neural stem cells marker including SOX-2, BMI-1 and Musashi-1. CD133, a 120 kDa cell-surface protein, is a marker for human neural progenitor cells [25, 162]. CD133+ cells isolated from human glioblastomas and clonally expanded can initiate new tumours when transplanted in the brain of adult immunodeficient mice showing also infiltrative properties [51, 153].

Brain tumours represent an enormous pool of proliferative and uncontrollable cells where the "cancer stem cell hypothesis" provides an interesting concept which could explain the resistance for most anti-cancer drugs.

Molecular mechanisms of invasion

Why are brain tumours so invasive?

The molecular machinery engaged in the progression of brain tumours is highly complex. Different mechanisms are in combination responsible for their growth, and we highlight in this review four main characteristics contributing to their invasive behaviour (Figure 1). In fact to be invasive, brain tumours cells need to:

1) Proliferate and expand, this might be due to mutations or loss of key regulators pathways and/or tumour suppressor genes.

2) Invade the healthy brain parenchyma, using different strategies of migration and adapted cell signalling with the surrounding extracellular matrix (ECM).

3) Maintain their source of energy by recruiting local blood vessels, and also by using angiogenic factors as a source of chemoattractant factors for the migration of cancer cells.

4) Be constantly supplied by a source of proliferative "cancer stem cells" that appear to localize within vascular niches.

These different characteristics have been the focus of extensive research, which is outlined below. A special attention is drawn to the close relation between blood vessels and gliomas cells and the concept that a "cancer stem cell niche" would supplie primary brain tumours with an unlimited pool of renewing cancer cells.

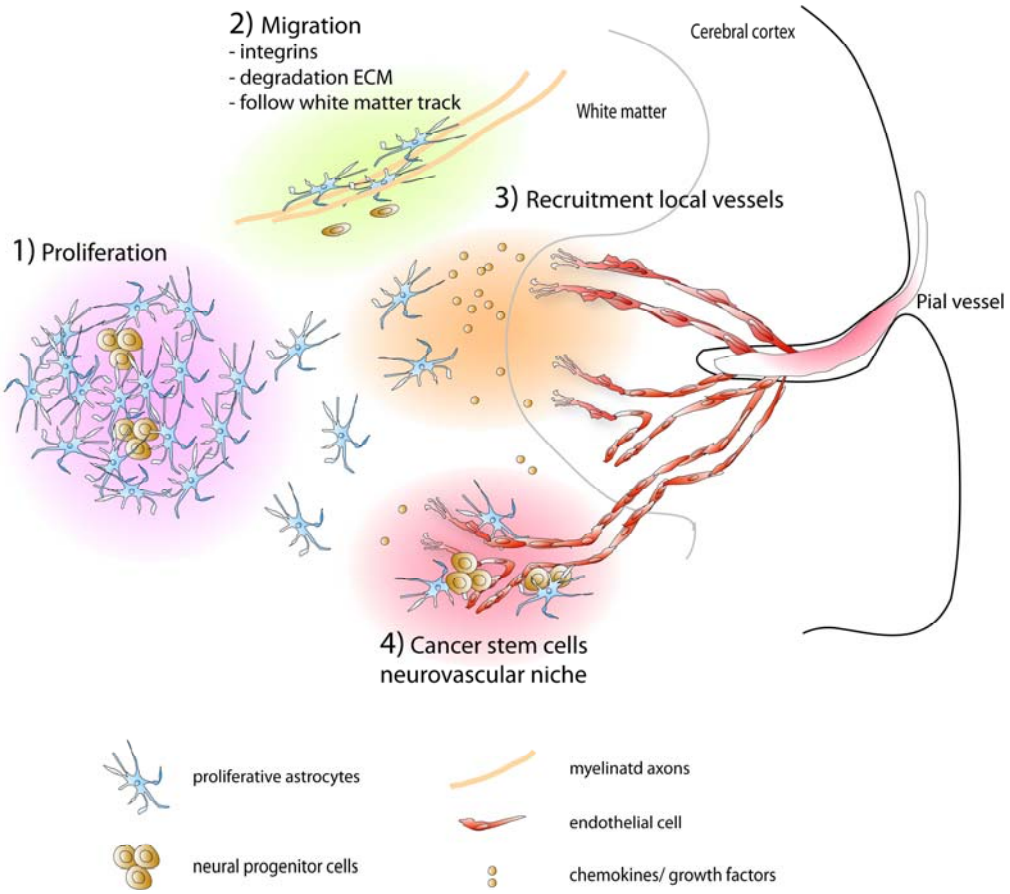

Figure 1: Schematic representation of four different main mechanisms contributing to brain tumour invasion

Brain tumour growth and invasion require a multistep process where different aspects can be analysed:

1) The molecular and genetic alterations (mutations) contributing for the growth and proliferation of brain tumour cells. 2) The different strategies that cancer cells have developed to migrate long distance in the healthy brain parenchyma. 3) The recruitment of blood vessels that is essential for the survival of tumour cells and which secretes chemoattractant factors. 4) The constant supply of cancer cells by the "cancer stem cell vascular niche".

1) PROLIFERATION / EXPANSION

Loss of function or inactivation of genetic pathways involved in cell cycle, can initate malignant tumours. In the central nervous gliomas can progress from a low grade too a high

grade and are then considered as secondary glioblastomas, but they can also develop primarily as high grade gliomas and are named primary glioblastomas.

Distinct genetic pathways, mostly identified as tumour suppressor genes, are involved in the initiation and progression of gliomas (Figure 2). These different pathways are explained briefly below as they have been already described extensively [9, 18, 38].

Initiation and progression pathways

A well known tumour suppressor gene is p53, mutated in 65% of secondary GBM and in 28% of primary GBM [120], while 50% of gliomas cells contain either mutated p53 or lack of p53 protein [86, 139]. The most common role of p53 is to induce cell cycle arrest, for DNA repair, in response to its damage. Cell cycle arrest is induced by p53 activation through the phosphorylation of the retinoblastoma (Rb) gene [49]. Interestingly in mouse fibroblasts, p53 also regulates cell morphology and migration. In fact both overexpression of wild type p53 and activation of endogenous p53 can inhibit filopodia formation suggesting a role in glioma cell migration [50], although invasion was not affected by p53 [171].

Other tumour suppressor genes such as the retinoblastoma gene *(RB)* have been linked to progression pathways of gliomas [9]. *RB* is an essential component of the regulation of the cell-division cycle and entry into DNA synthesis [147, 181]. Mutations in the RB pathway are seen in more than 80% of GBM and 50% of anaplastic astrocytomas [79, 145, 181], which suggests a strong link between gliomagnesis and alteration of this pathway.

Another well known suppressor gene is PTEN, involved in cell growth and survival by counteracting the effects of growth factors activation through the PIP2-PIP3 pathway [9]. Inherited mutations in PTEN cause pleiotrophic effects, including cancer predisposition. Somatic mutations in PTEN occur frequently as late events in brain tumours. Also, PTEN

mutations are seen at a lower rate in low grade gliomas [38], and can be seen in 25% of primary GBM and 4 % of secondary GBM [120].

Regulators of nervous system development as tumour suppressors

During development, pathways regulate the shape of the individual. Recent evidence suggest that these developmental pathways have a role as tumour suppressors [138].

The Wnt and sonic hedgehog (SHH) pathways share common features [9] including tumour suppressor in the cerebellum. The Wnt signalling pathway is involved in sporadic and syndromes medulloblastomas [40]. Involvement of Shh-Gli pathway in gliomagenesis has been suggested, since it regulates the development and abnormal growth of the dorsal brain. Moreover Gli is expressed in CNS tumours samples and tumour cell lines from GBM [138].

Furthermore, Notch signalling plays a considerable role during brain development and the maintenance of organisms, in particular stem cell proliferation [102]. The correlation between brain tumour growth and Notch expression is well documented for several tumour types. High levels of Notch 1 signalling have been shown in medulloblastomas [29]. Primary GBM have been shown to up-regulate Notch 3 [88], and expression of Notch 1 and several of its ligands is important for the survival and proliferation of astrocytomas cells [130]. Evidence also are growing for Notch signalling in brain tumour stem cells, where in an established U373 astrocytoma line, a subpopulation of cells that exhibit stem-like properties, increased rate of growth and high levels of Notch expression have been demonstrated [37, 150].

Growth factors

Different growth factors participate in the proliferation of brain tumours cells, with different level of expression. Through the activation of tyrosine kinase receptor (RTK), growth factors can play a significant role in the initial phase of glioma induction. Normal cells require

growth signals for survival and proliferation. Transmission into the cells is made by a group of transmembrane protein, the RTKs. Elevated expression of growth factors and their RTK receptors are found in astrocytomas, such as epidermal growth factor (EGFR) amplified in 36% of primary GBM and 6% in secondary GBM [120]. In low grade gliomas the earliest activation includes over-expression of platelet-derived growth factor (PDGF) ligands and receptors that causes an autocrine growth factor stimulation loop and inactivation of the p53 gene. Activation of PDGF receptor leads to multiple downstream signalling transduction pathways that induces cellular proliferation [11]. EGF has recently been shown to be crucial for the mitogenic regulation of stem cells from human gliomas [155]. GBM can also over-express insulin like growth factor binding protein-2 (IGFBP-2) [47], or bone morphogenetic protein in glioblastoma stem cells [103].

Molecules involved in DNA repair

Pathways involved with DNA repair might also contribute to the proliferation of brain tumours cells. The DNA-repair protein 06-alkylguanine-DNA-alkyltransferase (AGT) is encoded by the gene 06-methylguanine-DNA-methyltransferase (MGMT), located on chromosome 10q26 [70]. It protects against mutagenesis and malignant transformation. In tumours, AGT provides resistance to treatment with alkylation agents unless expression is lost by methylation of the promoter of the gene encoding AGT (MGMT). Overexpression of MGMT reduces the risk of carcinogenesis and the risk of mutations after exposure to methylating agent. MGMT-promoter methylation shuts off MGMT expression in tumours and increases responsiveness to chemotherapy [53]. Also, loss of MGMT is associated with increased carcinogenic risk and increased sensitivity to methylation agents, which then prolongs survival in glioblastomas patients treated with the alkylating agent temozolomide [70].

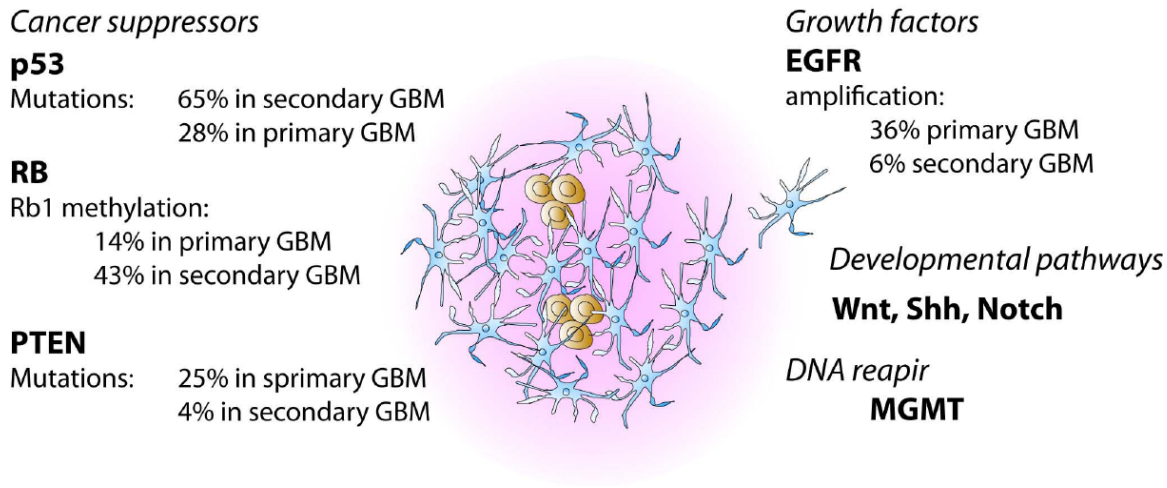

Figure 2: Schematic representation of the main pathways involved in brain tumour proliferation and expansion

Many different molecular pathways are involved in the intitation or progression of primary brain tumours. The well known cancer suppressor genes such as p53, RB and PTEN play an important role when mutated for the proliferation of cancer cells. Amplification of growth factor pathways, such as EGFR is also determinant for brain tumour expansion. Alteration in mechanism involved in central nervous system development could also play a critical role in gliomagenesis *(adapted from Ohgaki et al 2007, Ruiz I Altaba, 2002).*

2) MIGRATION

Malignant brain tumours have the vast potential to invade surrounding parenchyma making complete resection impossible. Molecular mechanisms developed for efficient invasiveness of cancer cells are various, and sometimes linked together for proliferation and migration [62]. Cell migration needs the succession of complex molecular events [45, 135], established in 5 steps: 1) protrusion of the leading edge, 2) cell-matrix interaction and formation of focal contacts, 3) recruitment of surface proteases to extracellular matrix (ECM) contacts and focalized proteolysis, 4) cell contraction by act myosin, 5) detachment of the trailing edge [45].

Cancer cells in order to migrate, attach to the ECM component, and create channels in the parenchyma by digesting the ECM [96]. In the brain most ECM components collagen, laminin and fibronection constitute the three principal ECM proteins of the blood brain barrier and are found preferentially in the perivascular spaces [140, 161]. Cancer cell invasion and migration are mediated by alterations in the expression of cell-surface molecules known as integrins, and release of proteases that remodel the ECM [31]. We review here the most important molecular mechanism that brain gliomas cells use to invade the parenchyma.

Integrins' attachment to the ECM

Integrins are a large family of glycoproteins that form cell surface heterodimeric receptors mediating the interaction with the ECM. Integrins are composed of an alpha and beta subunit [78], which combined will determine the ligands specificity [75]. Integrin's interaction with the ECM is crucial for progression and invasion of high grade gliomas [28]. The ECM consists of a network of molecules secreted by cells including ligands recognized by the integrins such as fibronectin, fibrinogen, Von Willebrand factor, collagen, laminin and vitronectin. Attachment between integrins and the ECM results in integrin clustering and remodelling of the cytoskeleton, leading to the establishment of focal adhesions between the cell and the ECM. Integrins become active by phosphorylation upon clustering [111], a way of regulating molecular interaction at these focal adhesion contacts [39]. Focal adhesion contacts are located at the leading edge of a moving cell [135]. The formation of clusters of integrins at focal adhesion are important for creating a "docking site" for focal adhesion kinase (FAK) leading to the assembly of cytoskelatal components (paxilin, talin, vinculin and alpha actin) that are crucial to create a link between growth factor and the cytoskeleton [164]. At the rear end of the cell, focal adhesion are disassembled, internalised and re-cycled, allowing detachment [135].

Evidence that brain tumour cells express integrins [56-58, 106, 114, 128, 129], and studies linking their over-expression to the invasiveness of brain tumour cells have flourished [1, 93, 117, 136, 141, 157]. Integrins such as Alpha(v)beta 3 and alpha(v)beta5 are expressed at in gliomas cells and the vasculature at the periphery of gliomas and corresponds to the tumour grade [13]

ECM around perivascular regions of the brain is rich in components like collagen, fibronectin and laminin and this is a specific site of localisation and invasion [112] for gliomas cells. In vitro studies have shown that the ECM components can affect the migration of gliomas cells. In fact, collagen, fibronectin, laminin and vitronectin promoted a differential migratory response using human glioblastoma biopsy sample grown as spheroids [113]. Laminin preferentially found around blood vessel, is the strongest stimulation for migration [48, 68, 112]. Interestingly, changes in laminin isoform from laminin-9 to laminin-8 produced by glioma has been shown to promote invasion [108]. When in contact with gliomas cells, the brain parenchyma can also change the composition of its ECM (laminin, collagen IV and fibronectin) [98].

FAK in integrin and growth factor mediated signalling

Another component of the integrin mediating signalling, the focal adhesion kinase (FAK) has shown a significant role in gliomas cell proliferation [141] [33] and invasive migration [77, 86, 122, 144, 149, 151]. It is associated with the formation and turnover of focal adhesion contacts [144] which then couples to the cytoskeleton via different complexes (paxilin, talin, vinculin and alpha-actinin) [62] [144] [160] [27].

Over-expression of FAK [67, 69, 141] has been described in brain tumours (high grade gliomas), could be linked with their development [69, 141], and is highly expressed in the infiltrative parts of the tumour [63, 67].

Moreover, FAK seems to play a pivotal role through growth factor stimulation. In fact over-expression of EGFR is common in high-grade gliomas. Increased migration of gliomas cells has been shown in response to activation of EGFR [73, 94, 125, 170]. Interestingly, gliomas cells lacking FAK do not respond to motility signals from EGF [86].

ECM derived from gliomas

Gliomas cells themselves can produce ECM components like tenascin-C and vitronectin. It was shown in a xenograft transplantation model that vitronectin seems to be over-expressed locally at the invading margins of gliomas together with tenascin whereas fibronectin is expressed diffusely throughout the tumour [13, 112]. Also laminin, fibronectin and collagen type IV were mainly produced by the host tissue and associated with blood vessels inside the tumour [112]. Expression of vitronectin could promote tumour cell invasion and be an important marker for tumour progression [28].

The other component of the ECM that is relevant for gliomas invasion is tenascin-C. It is expressed at the periphery of gliomas and produced by the tumour cells [13]. Tenascin-C is also expressed at high level in malignant gliomas [178], correlates with tumour grade [71], and tumour cell proliferation [12]. Staining for tenascin-C in the perivascular environment is correlated with a worse prognosis [104]. In high grade gliomas, expression of tenascin-C is correlated with angiogenesis and tumour cell proliferation [12]. These data suggest an important role for tenascin in the invasiveness of glioblastomas, and has been a molecular target for therapeutical strategies (Figure 3).

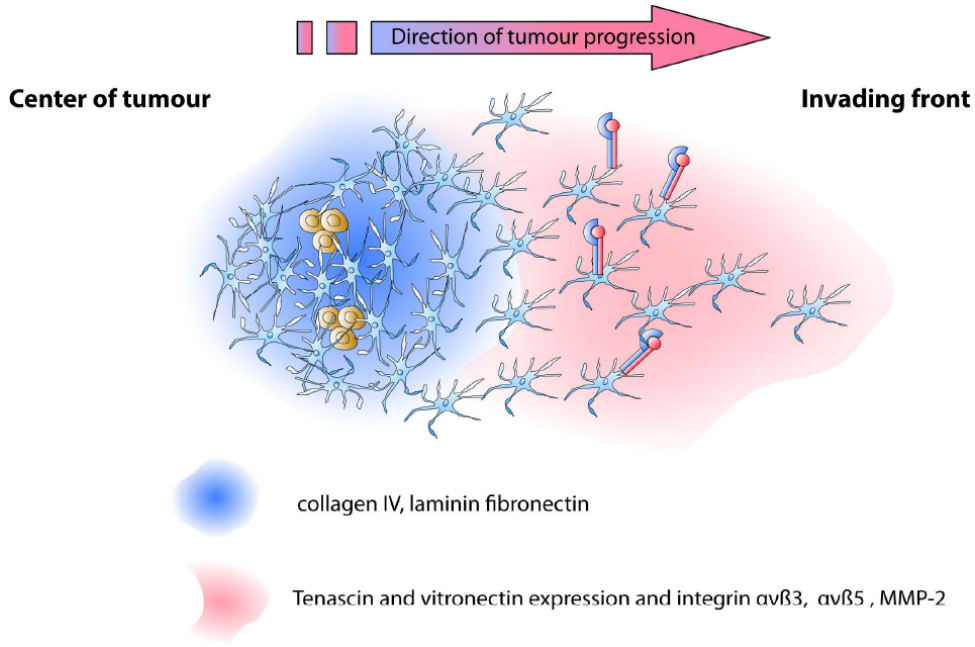

Figure 3: Role of gliomas derived ECM production in the invasive process.

ECM can be produced by gliomas cells. At the invading front of the tumour, tenascin and vitronectin are overexpressed along with the integrin receptor alphavbeta 3, alphvbeta5 and the proteinases MMP-2. In the center overexpression of collagen IV, laminin, fibronectin is more common. *(adapted from Bello et al, 2001, Mahesparan et al, 2003)*

ECM digestion

To advance through the brain parenchyma, cells digest the ECM, via proteases such as serine (urokinase-type plasminogen activator), cystein (cathepsin) and metalloproteinases (MMPs) to degrade the ECM [16]. These different pathways have been extensively reviewed elsewhere [131].

An important aspect in cell migration is the migration towards a gradient of chemoattractant molecules. It was described that soluble factors such as vascular endothelial growth factor, hepatocyte growth factor, but also epidermal growth factor and fibroblast growth factor as well as TGFs could act as chemoattractant factor and also directly modulate the expression of

proteases such as MMPs on the gliomas cell surface, therefore directly enhancing the molecular mechanisms important for cell migration [118].

In overall, migrating brain tumours cells use to their advantage the complex machinery of any cells to traverse a complex tissue structure, composed of a dense matrix where mechanism providing strong attachment and digestion are critical for invasiveness.

3) ROLE OF ANGIOGENESIS AND ANGIOGENIC INDUCTION IN GLIOMAS INVASION

Angiogenesis, the process by which new capillaries sprout from pre-existing blood vessels by endothelial cell proliferation and migration is a key aspect of brain tumour growth and invasion [83]. Research is very active in determining the impact of angiogenesis on tumour development and the reciprocal influences of tumour products on the microvasculature [14]. Angiogenesis is flourishing in glioblastomas compared to low grade gliomas and an enormous hope has been placed in the last decade for anti-cancer therapies targeting angiogenesis. Angiogenesis and brain tumour invasion are co-dependant processes, in fact, induced angiogenesis in gliomas has been shown to correlate to gliomas cells migration in vivo [163].

Both endothelial and gliomas cells migrate by modifying the adhesiveness with the ECM, expressing new adhesion molecules and degrading the ECM components by secreting active proteases. Cancer cell secrete factors that recruit blood vessels from the surrounding parenchyma. Key molecules involved in cancer angiogenesis have been the focus of extensive research. Briefly, angiogenesis represents a balance between proangiogenic stimuli (FGF-2, PDGF, VEGF, IL-8, HGF/scatter factor, granulocyte colony-stimulating factor and angiopoietin) and angiogenic inhibitors (TS1, angiostatin, IFN-alpha, metalloproteinase inhibitors, TGF-beta and endostatin) (Figure 4A). In cancer, proangiogenic stimuli dominate,

including FGF-2, VEGF, PDGF in particular VEGF expression is upregulated, resulting in activation of the "angiogenic switch" [14, 42] and therefore neovascularisation in astrocytic tumours.

Some of these angiogenic factors might act also directly on tumours cells and facilitate their invasion and migration. In this chapter, we focus precisely on how blood vessel and their relevant angiogenic pathways contribute significantly to brain tumour cell invasion.

Angiogenic factors involved in brain tumour cell migration

Role of HIF

Fast growing tumours suffer from hypoxia due inappropriate blood supply. Hypoxia and the hypoxia inducible factor-1 (HIF-1) are critical in glioma growth and angiogenesis, and HIF-1 is believed to be a proangiogenic "master switch" [92]. Hypoxia might also trigger directly tumour cell migration [127]. In this field, an emerging concept the "go or grow" hypothesis suggests that cell division and cell migration are temporally exclusive events and that tumour cells have to choose between proliferation and migration. Some external stimuli, like hypoxia could push the cells towards a migratory behaviour therefore triggering molecular switch that will set up the complex molecular mechanism necessary for cell migration [26].

HIF-1 is a transcription factor that regulates oxygen homeostasis in response to changes in oxygen levels in normal and tumour tissue. HIF-1 is ubiquitously expressed and highly conserved heterodimeric basic-helix-loop-helix-PAS transcription factor composed of an alpha and beta subunit. When cell oxygen level is low, the HIF-1alpha subunit increases which determines HIF-1 activity and activates a succession of genes containing which hypoxic response elements (HREs) [92]. One of these genes is the well known angiogenic factor VEGF, another one is CXCR4, a chemokine receptor for stromal cell-derived factor 1 (SDF-1) alpha, which is an important molecule associated with tumour progression.

A direct role for HIF-1 in gliomas cell migration has been shown. Typical features of glioblastomas are palissades, areas of focal necrosis where oxygen level is low. HIF-1alpha is upregulated in palissading cells around the areas of necrosis and this correlates also with the localized overexpression of VEGF and CXCR4 in the same areas [132]. In vitro, gliomas cell migration can be increased under hypoxic condition and is inhibited by CXCR4 inhibitors [178]. Also, silencing HIF-1 alpha with siRNA significantly suppresses glioma cell migration in vitro [46].

Another regulation of HIF-1alpha is through genetic alterations leading to HIF activation in gliomas. Both the activation of growth factors (EGFR) and the loss of tumour suppressor function (p53, PTEN) common in gliomas can affect HIF expression. Mutatant EGFR by can activate the PI3K pathway which increases HIF-1alpha, increasing therefore downstream gene such as VEGF [24]. PTEN loss of function has been shown to experimentally increase HIF-1alpha expression and tumour vascularisation in gliomas (Figure 4A) [92].

Role of VEGF

VEGF, a strong angiogenic factor, stimulates the migration of endothelial cells. It might be also directly involved in the migration of gliomas cells. The biological effects of VEGF-A are mediated via two receptor kinase VEGFR-1 and VEGFR-2 [42]. In gliomas, VEGF is strongly expressed at the leading edge of parenchymal infiltration [107] and in higher grade gliomas, concomitant with the expression of VEGF-C [85]. VEGF also upregulates the expression of MMP-2 and MMP-9 [137] and to down regulate TIMP-1 and TIMP-2 [100]. VEGFR-1 is expressed on gliomas cells, and VEGF inhibition can lead to a decrease of tumour cell proliferation and migration, affecting therefore tumour invasion [72].

Role of FGF-2

FGF-2 is also known for a strong angiogenic potential has a role also in glioma cell line migration [20], and proliferation of glioma cells via an autocrine loop [5]. Interestingly inhibition of FGF-2 and FGFR activity in C6 glioma cells reduces tumour growth [8].

Role of MMPs

Matrixmetalloproteinases (MMPs) essential for endothelial cell migration [99], play an important role in angiogenesis [52], but also in glioma cells migration [46, 179]. Interestingly, MMP-2 is highly increased in gliomas. In gliomas cell lines MMP-2 downregulation by siRNA is correlated with a significant decrease in invasion through matrigel. Also a decreased migration from tumour spheroids transfected with adenoviral vector expressing siRNA against MMP-2 was been seen with the same model [91]. Another example of the relation between blood vessel and the invasion of gliomas cells has been shown in a model of genetically engineered MMP-2 knockout GBM, showing an increased vascular density associated with VEGFR-2 activation and enhanced vascular branching and sprouting. Interestingly, despite the high vascular density, tumour cells were more apoptotic [36].

Endothelial cells and glioma cells might respond differently to chemoattractant factors. Using modified Boyden chamber assays, chemoattractant effects were studied on three different glioblastoma cell lines and on human cerebral microvascular endothelial cells. SH/HGF was the strongest chemotactic factor on the three glioblastoma cell lines, and induced up to 33 fold increased migration. TGF-alpha showed the second strongest effect with 17 fold stimulation followed by FGF-1. EGF, FGF-2 IGF-1 TGF-beta1 and TGF-beta2 were chemotactic for one or two cell lines, whereas PDGF, VEGF, PTN and MK had surprisingly no effects, on this migration assay [20].

The relevance of the perivascular environment

Many of the factors relevant for angiogenesis are crucial for the invasion of brain tumour cells, thus emphasizing the relevance of the close inter-dependence between blood vessels and tumour cells for the growth and invasion of brain tumours. Cancer cells might use the perivascular environment as a physical route of migration following blood vessels covered with laminin [61, 93]. Most importantly, in the microenvironment of blood vessels, growth factors secreted for the migration of endothelial cells and the sprouting of new capillaries might be beneficial for the growth of cancer cells [167]. The micro-vascular environment contains all the components of the extracellular matrix that is important for the growth and controlled migration of invasive endothelial cells [22, 168], as well as cancer cells. It is known that in glioblastomas, cells are located predominantly around blood vessels, were they proliferate [158]. Electron microscopic analysis of GBM showed that vessels can be totally surrounded with neoplastic cells [6] (Figure 4B).

In a model of slice cultures from rat forebrain, transplanted C6 glioma cells extensively infiltrated the brain by migrating along the abluminal surface of blood vessels. Interestingly, the majority of glioma cell divisions took place near vascular branch points, suggesting that mitosis was triggered by local perivascular microenvironmental cues [41].

Moreover, GL261 gliomas cells on normal brain of live mice in real time, invade the perivascular space often in nest close to multiple capillary structures where microvessels run parallel to each other, capillary loops or glomeruloid like bodies and also dilated capillaries [172].

In another study, glioma cell suspension transplanted onto sections of human brains showed to spread rapidly on blood vessels and arachnoid tissue. The predominant matrix proteins in small vessels are laminin, collagen type IV and fibronectin. Adhesion to blood vessels on brain sections can be inhibited to 50% by anti-integrin beta 1 [54].

SDF-1alpha seems to be involved in the migration of glioma cells around blood vessels, as it was shown that on glioblastoma samples, SDF-1alpha immunoreactivity was present in blood vessels, whereas CXCR4 immunoreactivity was observed in glioma cells surrounding the blood vessels [177]. During tumour progression, high degree of neovascularisation occurs, and some of the particular feature of these tumour vessels is that they lack a blood-brain barrier which leads to a leaky vasculature. It has been suggested that this leaky vasculature imporoves the migration of tumour cells [4].

Homeostasis of the perivascular space is a crucial aspect for cancer cell survival, and factors regulating the cell/cell interaction in this microenvironment might be a target for anti-cancer therapies.

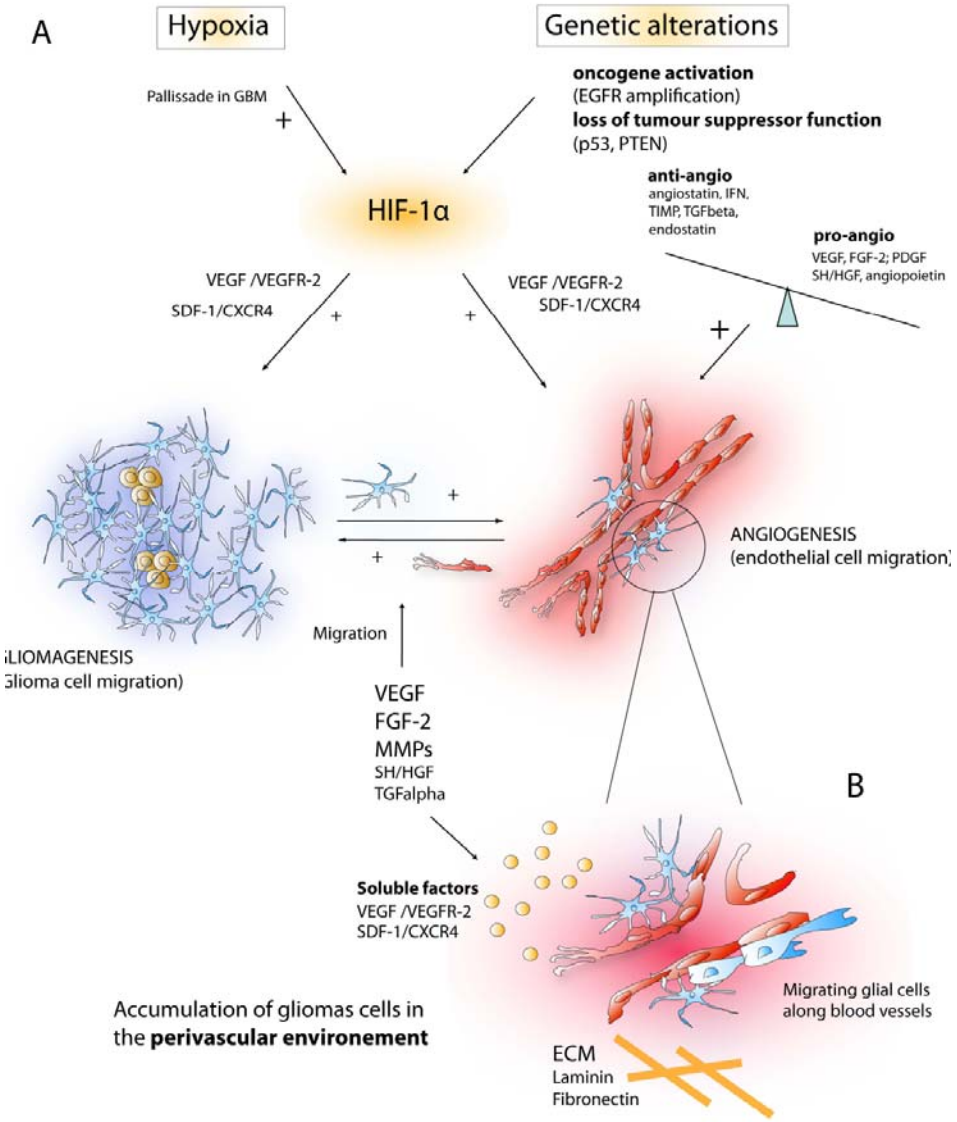

Figure 4: The role of angiogenesis and angiogenic factors in brain tumour cell invasion.

Angiogenesis and tumour cell invasion are codependant pathophysiological processes playing a pivotal role in glioma development and invasion, since the start of tumour development. Angiogenesis is induced by a balance between the activation of pro-aniogenic factors or anti-angiogenic factors. (A) HIF-1alpha is considered as a proangiogenic "master switch" and is induced in brain tumours by hypoxia but also directly by genetic alterations such as oncogene activation (EGFR amplification) and loss of tumour suppressor function (p53 and PTEN). (B) An important aspect of gliomas progression and invasion is the perivascular environment where the ECM is the most enriched; the interdependence between cancer and endothelial cells is the closest through

soluble factors secreted in the perivascular environment. Moreover, gliomas cells are seen to migrate away from the tumour core along blood vessels. *(adapted from Kaur, B et al 2005, Clarke et al 2001, Kawataki, et al 2007)*

4) THE ROLE OF CANCER STEM CELLS AND VASCULAR NICHES

The concept that a small population of cells, the cancer stem cells are at the origin, and maintain the aggressive behaviour of cancers, started from studies on leukemia [101] and has been since well accepted [133]. Cancer stem cells that can start new tumours through serial transplantation in mice have been identified also from glioblastomas [153]. Although the exact molecular pathways participating in the maintenance of stem cells are unknown, it appears that the role played by their close microenvironment is critical [84], and cancer stem cells need to be maintained in a "cancer stem cell niche" [154].

In the adult nervous system, neural stem cells are maintained in niches and their close relation with vessels play a determinant role for heir survival [3, 34, 116]. In brain tumours formation also, the close relation between vessels and cancer stem cells in a cancer stem cell vascular niche complex seems to play an important role [55, 165, 175].

Recent data suggest that the molecular steps in glioblastoma formation can be partly similar to neurogenesis [126], reinforcing the concept that brain tumour formation could result from the dysregulation of neural stem cells. In fact, common signalling pathways like Olig2, a central nervous system restricted transcription factor [105], or excessive PDGF activation [82] are important for the proliferation and survival of both normal and cancer neural stem cells. Moreover, deletion of NF1 and p53 from neural stem cells in mice leads to the development of glioma in the subventricular zone [180](Figure 5).

Evidence for cancer stem cell vascular niche in primary brain tumours

Evidence of a functional relationship between the tumour vasculature and glioblastoma stem cells was not yet provided, until it was shown that stem cell like glioma cells (CD133+),

isolated from glioblastomas specimen generated tumours associated with widespread angiogenesis when transplanted in the brains of immunocompromised mice, compared to the transplantation of non-stem cell like glioma cells (CD133-). This effect was confirmed in vitro where stem cell like glioma cells induced stronger tube formation and endothelial cell migration compared to non stem cell like glioma cells when co-cultured with endothelial cells [10]. A further study localized CD133 + brain tumour cancer stem cells in the perivascular niches as dispersed single cells and in pseudopalissade formation around necrosis [23].

As compared with neural stem cells, the co-dependence between brain tumour stem cells and endothelial cells was further demonstrated. It was clearly shown that nesting positive brain tumour cells associate with the tumour vasculature in glioblastoma and that CD133+ brain tumour cells interact physically with endothelial cells in culture, maintaining them in a self renewing and undifferentiated state. Moreover, co-transplantation of brain tumour stem cells and endothelial cells into immunocompromised mice, showed that endothelial derived factors also increase the initiation and growth of tumours in the brain [21].

The concept that cancer stem cells vascular niche exists in gliomas and provide unlimited pool of abnormal and invasive glioma cells and the molecular mechanisms involved in this process need further studies, but could be a determinant key target for anti-gliomas therapies.

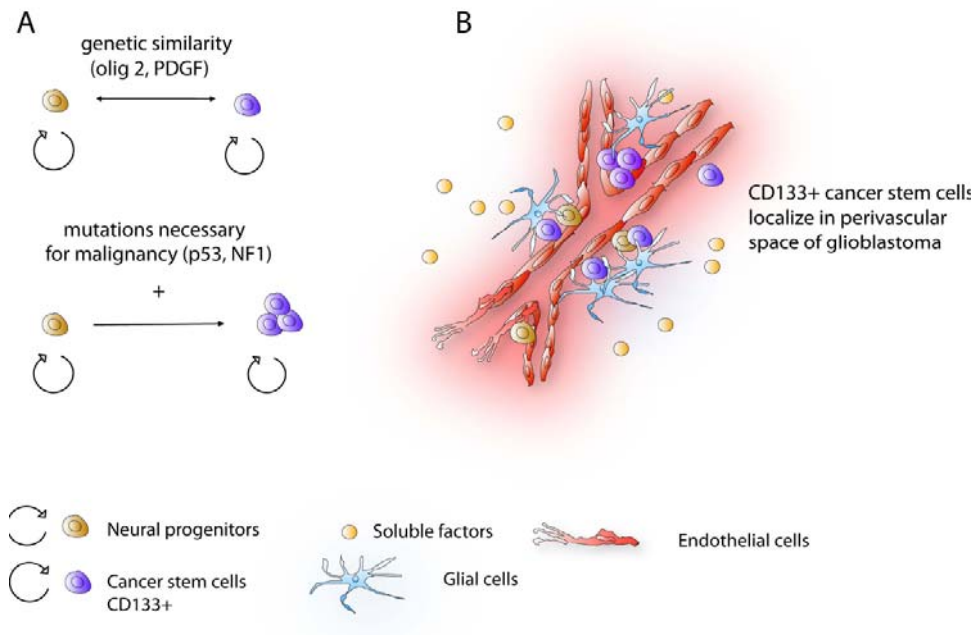

Figure 5: Role of cancer stem cell niche in gliomas invasion

(A) Genetic similarities exist between neural progenitors and cancer stem cells. It is suggested that crucial mutations necessary for malignancy (p53) will change neural progenitor cells into gliomas cancer stem cells that are able to produce glioblastomas when transplanted into the cortex of nude mice.

(B) Gliomas cancer stem cells are CD133+ and are found to be in the perivascular space, like endogenous neural progenitors cells. It is suggested that this close interaction between endothelial cells and cancer stem cells constitutes the "cancer stem cell vascular niche" that is crucial for the survival, proliferation and progression of brain tumours. *(Adapted from Jandial et al 2008, Veeravagu et al 2008, Ligon et al 2007, Jackson et al 2006)*

CHAPTER II: BRAIN METASTASIS

Metastasis from a primary cancer is a major cause of morbidity and mortality in human malignancies. It has been reported that between 20 and 40% of patients with systemic cancer will develop metastasis involving the CNS. In adults, metastasis to the brain most commonly comes from primary tumours of the lungs (50-60%), breast (15-20%) [169], skin (melanoma) (5-10%) and gastrointestinal tract (4-6%) [119]. The incidence of brain metastasis is estimated, by some studies, to be at least 10 times higher than primary brain tumours. Patients suffering form brain metastasis have in general a very short life expectancy, although aggressive surgical and oncological treatment is given.

In metastasis formation, an important aspect is the haematogenous dissemination form the primary cancer but also the fact that metastatic cells need to adapt, grow and recruit blood vessels in a different organ. In this review we will focus on the current knowledge of the principal molecular and genetic processes responsible for the patho-biology of brain metastasis seeding and invasive characteristics, with a special emphasis to the mechanisms of invasion that are different from primary brain tumours.

Mechanisms of metastasis seeding

The metastatic process is not random as suggested earlier [123], but is a highly selective process consisting of a series of events [119], described has the "metastatic cascade" [43, 124]. It involves cancer cells to (1) escape from the primary tumour by invasion of the surrounding tissue, (2) reach the brain vasculature and surviving into the blood stream in a process called intravasation, (3) attach to endothelial cells, (4) extravasate into the brain parenchyma (5) proliferate and invade at the destination with the initial formation of the "premetastatic niche" and induction of angiogenesis (Figure 6) [44, 110, 159]. In the brain,

metastases tend to localize in the areas of the grey-white matter junction and in the "watershed area" of the arterial circulation, the zones on the border of the territories of the major circulation. This distribution is due to the progressive decrease in the size of the blood vessel in these areas: terminal arterioles present in this site acts as trap emboli [159].

Many different aspect of the metastatic cascade are determinant for the aggressive behaviour of these tumours, but we will highlight here one aspect, the formation of the "premetastatic niche" and its dependence on angiogenic molecule for the initiation of a micrometastases formation.

Formation of the premetastatic niche

An interesting concept in the formation of brain metastasis is the development of the "premetastatic niche". Once attached to the endothelial surface, cancer cells extravasate into the brain parenchyma. This process is regulated by molecular events, involving the adhesion to the ECM, the control of cell migration which depends on local cues determined by the perivascular microenvironment. It is also suggested that hypoxia and local inflammation could help the migration of metastatic cells through the blood brain barrier and create a favourable environment for the formation of the "premetastatic niche" [89, 90].

New evidence show a role for haematopoietic progenitor cells in the formation of the "premetastatic niche". It was shown that bone derived haematopoietic progenitor cells (HPCs) that express VEGFR-1 localize to tumour-specific pre-metastatic sites, and form cellular clusters before the arrival of the tumour cells. Interestingly, it was also shown that blocking VEGFR-1 function using antibodies or by the removal of VEGFR1+ cells from the bone marrow of wild-type mice blocked the formation of these pre-metastatic clusters and prevented tumour metastasis [90].

Interestingly, after reaching the premetastatic niche, cancer cells can reside in a dormant state, which is mediated by inhibition of neovascularisation. The balance between proangiogenic factors (VEGF; platelet-derived growth factor, endothelial growth) and anti-angiogenic factors (thrombospondin-1, platelet factor IV), determines the course of disease progression. Removal of inhibitory antiangiogenic factors can lead to the growth of metastases from dormancy [124, 142].

The above descriptions highlight new emerging evidence of the importance of the "premetastatic niche" formation in destination organs by hematopoietic derived cells. Some have suggested that the dormant state of metastatic cancer cells is analogous to that of cancer stem cells, both require a permissive milieu, to acquire their proliferation potential (Figure 6).

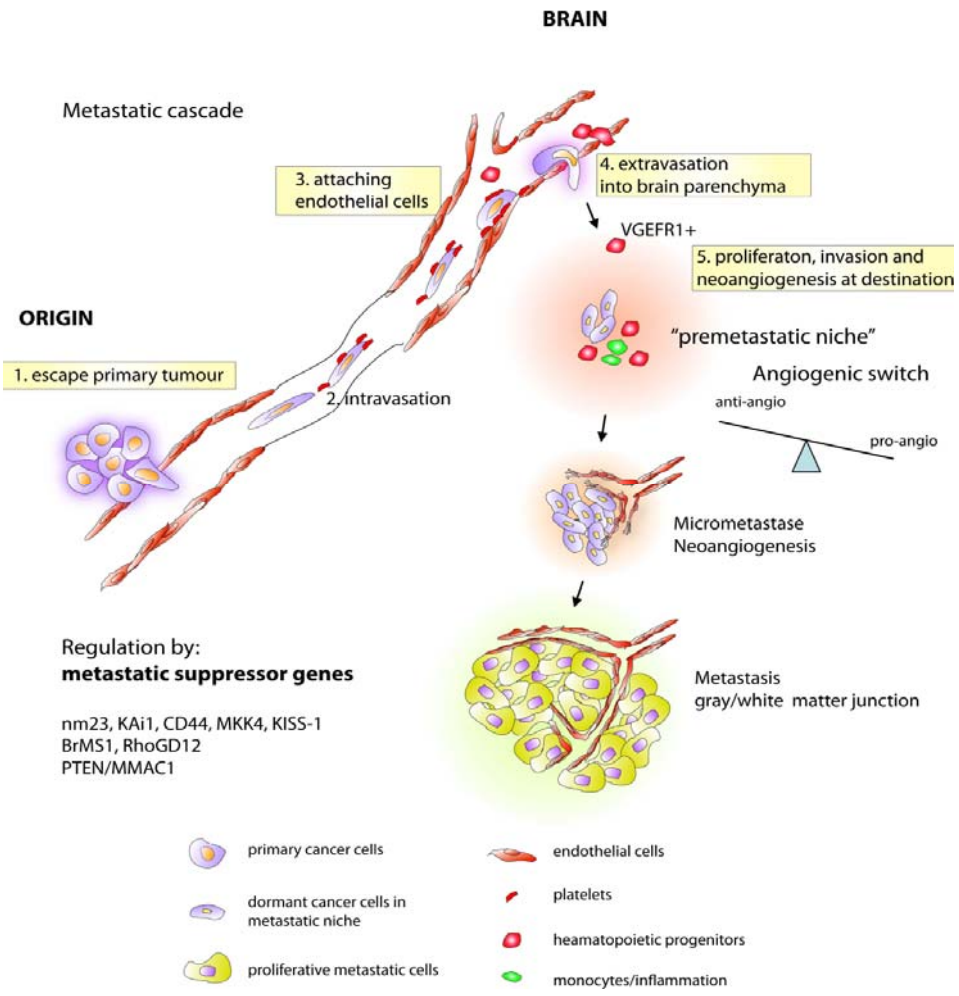

Figure 6: Metastasis formation in the brain

At the site of the origin, cancer cells will: **1)** escape from the primary tumour into the blood capillaries. **2)** Then through a process called intravasation, platelets aggregate cancer cells which once reaching brain capillaries clog at the end of them, **3)** attach to the endothelial cells and then **4)** extravasate into the brain parenchyma. Once into the brain parenchyma, they **5)** proliferate and invade the brain parenchyma, inducing strong neoangioenesis. This last event follows a multiple step process, with the formation of the **"premetastatic niche"** composed of only a few cancer cells, hematopoietic progenitors and inflammatory monocytes. After induction of the angiogenic switch, micrometastase develop with their subsequent growth and formation of an invasive metastasis, usually at the grey/white matter junction. *(Adapted from Santarelli, 2007)*

Intracerebral metastatic invasion

Brain-metastasis interface

The borders between a brain metastasis and the surrounding brain parenchyma are usually well defined. Compare to gliomas which will diffuse and infiltrate the brain parenchyma, metastatic tumours are usually sharply separated from the surrounding brain tissue and easy to separate during microsurgery. Nonetheless, the surrounding brain tissue reacts from the presence of the metastatic tumours, mainly by recruiting blood vessels form the pia matter at the tumour/brain interface, this resulting in a series of pathological and biochemical changes [119]

Local tumour invasion

Following almost the same rules of primary brain tumour invasion, the local invasion of metastasis into the brain is a multifactorial process including cell motility, adhesion and proteolytic remodelling of the extracellular matrix. Brain invasion requires paracrine interactions between brain stromal, endothelial cells and the invading metastatic tumour cells. Degradation of the ECM is known to help tumour invasion by clearing a pathway for the

invading tumour cells. The proteolytic activity is concentrated on the advancing edge of the invading tumour cell [173]. Some have also suggested that the proteolysis activity might release factors from the ECM, which will activate proliferation and angiogenesis [119].

E-cadherin-catenin complex is an important mediator of cell-cell adhesion and is crucial for maintenance of normal and malignant tissue architecture. Reduced expression of the complex has been associated with tumour invasion and metastasis [19]. Interestingly in a study the quantitative expression of E-cadherin was used to predict tissue specific metastasis [30].

Integrins also have a role in local brain metastasis invasion. Blocking of integrin alpha3beta1 in animal model, using a human non-small cell lung cancer cell line, significantly decreased brain metastasis [176].

Neurotrophins (NT) are also important since they stimulate brain invasion. In brain metastatic melanoma cells, NT stimulates invasion by enhancing the production of ECM proteolytic enzymes [32], such as heparinase able to destroy both the local ECM and the blood brain barrier [115].

Regulation of the urokinase type plasminogen activator (uPA) is strongly linked to cancer invasion and metastasis. High amounts of uPA were found in glioma and metastasis whereas low amount were found in low grade glioma. The expression of uPA also correlates with malignant brain tumours and aggressive behaviour [17].

MMPs are also involved in the invasion of brain metastasis. In a study, all metastatic brain tumours were over-expressing MMP2 [81], and in an other series MMP9 was shown to be upregulated also in all brain metastasis [7].

Brain metastasis and neoangiogenesis

As for primary brain tumours, angiogenesis is necessary for continuous metastatic tumour growth. The onset of angiogenesis within small clusters of tumour cells, the “premetasatic

niche" is limited by the tissue diffusion distance, which is 0.2 mm. The onset of angiogenesis within small clusters of tumour cells is known as the "angiogenic switch" which is directly influenced by a balance between pro and anti-angiogenic molecules, where MMP-9 has been shown to be one of the initiators of this switch during carcniogenesis [15]. Further evidence of the direct role of angiogenic factors like VEGF in brain metastasis have been reported in a study were in an animal model, raised VEGF expression contributed to the ability of breast cancer cells to form brain metastasis [95, 121].

Molecular regulation of metastatic growth (metastasis suppressor genes)

Metastasis formation is a rather organ specific event and the multistep cascade of metastasis formation is tightly regulated by a cascade of genes. In this field, it has been suggested that metastasis suppressor genes (MSGs) could exists, and suppress the metastatic invasion at any point of the cascade. These genes are distinct from oncogenes, and include Nm32, KAI1, CD44, MKK4, Kiss-1, BrmS1, RhoGD12 and PTEN/MMAC1 which have been previously reviewed (Figure 6) [119].

Briefly, studies have shown that a reduction of Nm23 gene is associated with increased metastatic potential in several human malignancies. For the formation of brain metastses patients with cutaneous melanoma and a low expression of nm23 appear to be more at risk [143]. KAI1, is a potential marker for human breast cancer progression and is inversely correlated with the progression of the disease [174]. Also CD44 an integral membrane glycoprotein is upregulated in 48% of brain metastasis [65]. Kiss-1 a gene encoding for metastatin is a potent inhibitor of cell motility, leading to suppression of cell growth and antimetastatic activity [76]. BrMS1 is found on chromosome 11, and is expressed primarily in melanoma and breast cancer cells. It restores the normal gap junction phenotype, which helps the maintenance and communication within cells within the primary tumour, thus reducing

disseminated tumour cells growth [146], which is shown in vitro by the expression of BRMS1 that inversely correlates with the metastatic potential of melanoma cells [148]. Moreover, PTEN or MMAC1 (mutated in multiple advanced cancer) have been suggested to be involved in the formation of metastasis. In a study of 56 brain metastasis loss of heterozygosity (LOH) was seen in 67 % of brain metastasis derived from lung and 64% from brain metastasis derived from breast cancer. Nontheless, the mutation rate of PTEN/MMAC1 was low, with only 14% of PTEN/MMAC1 mutation in brain metastsis studied [64]. The demonstration that metastasis suppressor genes exists gives hope towards a therapy aiming to block the metastatic process at any steps of the metastatic cascade.

CONCLUSION

We have highlighted in this review different aspect for the growth and invasion of malignant brain tumours especially high grade gliomas, as well as brain metastases. Many reasons determine the aggressivity of brain tumours and metastases in the brain. Mutations in tumour suppressor genes might be the initiating step launching the activation of a multistage molecular cascade that is involved in: 1) proliferation, 2) migration, 3) recruitment of blood vessels and 4) maintenance proliferative “cancer stem cells” within the microenvironment of vascular niches. These many different characteristics of brain tumours contribute to the uncontrollable machinery that makes brain tumour cells especially high grade gliomas cells so invasive and destructive. Most importantly, they might be the target of adjuvant therapies aiming to control the progression of the disease.

A characteristic aspect that is common to both high grade gliomas and metastases is the crucial role played by the initiation of angiogenesis or “angiogenic switch” but in particular the key function of the microvascular environment for the proliferation, migration and

maintenance of cancer cells. In glioblastomas, cancer cells are located in the perivascular space that seems to be also the favourite location for cancer stem cells that are maintained and proliferative in these glioblastoma vascular niche complex sharing similarities to the physiological neurovascular niches of the gemrinative zone of the adult brain. In brain metastasis also, the initiation of the "premetastatic niche" depends on haematopoietic progenitors and inflammatory monocytes and the further growth of the micrometastasis to a full size metastasis depends on angiogenesis initiation, triggered by proangiogenic molecules. Anti-angiogenic therapies have been a hallmark in cancer research for the past ten years. The possibility that specific molecular pathways could be determinant for the maintenance and proliferation of cancer stem cells in the microvascular environment of brain tumours is an interesting target for future therapies

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