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MECHANISMS OF TUMOR CELL INVASION AND ANGIOGENESIS IN THE CENTRAL NERVOUS SYSTEM

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1. ABSTRACT

Despite extensive progress in characterizing the genetic events involved in the development of gliomas, the cellular origin and the defined molecular mechanisms that lead to their occurrence are still unclear. It is known that tumours are of monoclonal origin. This is contrasted by the fact that gliomas frequently express features of different glial cell lineages. With the identification of pluripotent neural stem cells and the growth factors that control neural cell development, we are now making early inroads towards understanding glial cell migration as well as the neural cell plasticity within the adult central nervous system (CNS). Gliomas share several fetal antigens with immature brain cells. It is therefore tempting to speculate that the migration of neural precursor cells actually represents the normal counterpart of glioma cell migration. The migratory behavior of gliomas may be due to a predetermined interplay between normal brain tissue and the migrating cells, where the brain represents a permissive tissue for guiding cells with certain phenotypic traits to migrate along specific anatomical structures.

Malignant progression is also accompanied by extensive angiogenesis which is especially prominent in glioblastoma multiforme (GBM). For cell proliferation to take place, several cell signaling cues mediated by specific growth factors are shared between the glioma cells and the endothelial cells while others are unique for endothelial cells. Therefore the endothelial cell compartment represents a promising target for novel therapeutic strategies including gene therapy and cell-based therapies.

2. INTRODUCTION

Malignant brain tumours are known for their diffuse infiltrative growth within the CNS, and at the time of diagnosis, most patients have disseminated tumor cells far beyond the areas that are accessible by surgical intervention. Despite an extensive local invasion, the tumours only rarely metastasise. The present work will address three important hallmarks that characterise glioma development and progression. The first is related to their cellular origin, the second to the mechanisms that determine their invasive characteristics, and the third to the mechanisms that regulate their blood supply. Brain tumor development should be regarded as a complex biological system where numerous intra as well as intercellular signaling networks act in concert. Several of these are dealt with in detail in other reviews within this special issue, as for instance aspects of gliomagenesis (1) angiogenesis (2) and the involvement of proteases during glioma cell invasion (3). During the last decade it has become clear that the cancer genome and the biological events that lead to a progressive disease is extremely complex. In this context, focus will be given towards describing global portraits of

cellular networks rather than detailed molecular events within, which to a large extent will be covered by other reviews within this volume.

In addition this review will focus on new therapeutic strategies for gliomas, such as antiangiogenic therapy and cell-based delivery systems of proteins with a therapeutic potential.

3. THE ORIGIN OF GLIOMAS; PERSPECTIVES FROM DEVELOPMENTAL BIOLOGY

Extensive cell proliferation, migration and loss of contact inhibition are key features of malignant neoplasms that are also shared by immature neuroectodermal cells during CNS development. There are several ways through which the principles of developmental biology may provide novel insight into fundamental mechanisms governing glioma progression. The development of mature glia cells starts with the formation of multipotent neural precursors present within the ventricular and subventricular zones of the CNS. Their existence has been documented by retroviral labeling of neuroepithelial cells by the lac*Z* reporter gene or by Bromodeoxyuridine labeling of proliferating cells (4, 5, 6, 7, 8). These studies have revealed that the progenitor cells have a short cell cycle time. The dividing cells give rise to new progenitor cells that are destined to terminally differentiate. It is well known that such germinal centers persist in the adult brain, especially within the subventricular zone of the lateral ventricles and in the subgranular layer of the hippocampal dentate gyrus (9, 10, 11, 12). The progenitor cells that are localized in these areas may differentiate into radial glia, which can divide asymmetrically to give rise to neurons, glia and perhaps, other cell types. In mammals, the radial glia disappear perinatally as they are thought to differentiate into astrocytes (13). From extensive studies both *in vitro* and *in vivo*, two distinct neuronal-glial cell lineages have been identified. In the Type-1 astrocyte (T1A) lineage, T1A precursor cells develop into mature astrocytes within the adult brain (14). In the oligodendrocyte-Type-2 astrocyte (O2A) lineage, the O2A progenitors differentiate into oligodendrocytes, but may also differentiate into Type-2 astrocytes (T2A) *in vitro*. At present, it is not clear if the O2A progenitors can differentiate into T2A cells *in vivo*, but both T1A and O2A progenitor cells have been demonstrated to be present in the developing CNS (15, 16, 17, 18, 19, 20, 21). Presumably, the O2A progenitors may be capable of transforming into oligodendrogliomas, astrocytomas or mixed tumours. By applying techniques previously utilized to study glial progenitor cells, human glioblastoma-derived cell populations can be isolated that express many properties that otherwise uniquely reflects O2A progenitor cells (23). Furthermore, lineage tracing studies in animals have been used to correlate specific cell characteristics with the histology of gliomas. It has been shown that undifferentiated cells are more sensitive to oncogenic effects of certain cell signaling abnormalities than are differentiated cells (24).

3.1. Astrocytes may show stem cell characteristics

Self-renewing cells from the subventricular zone can be propagated *in vitro* in the presence of high concentrations of basic fibroblast growth factor (bFGF) and epidermal cell growth factor (EGF) (25, 26, 27). If the growth factors are removed, the cells may differentiate into neurons, astrocytes and oligodendrocytes. These *in vitro* progenitors have been termed neural stem cells (28, 29, 30). Even though these studies have revealed a common progenitor giving rise to several neural phenotypes *in vitro*, it is still debatable whether these cells correspond to the initial precursors of glia and neurons *in vivo*. Recent studies have revealed neural stem cells within the subventricular zone that show phenotypic features previously attributed to mature glia. For instance, cells with an astrocytic phenotype can in the presence of EGF produce progenitor cells that differentiate into neurons and glia (31). Furthermore, astrocytes of the subventricular zone may in the presence of EGF, bFGF and fetal calf serum behave as neural stem cells (31). These *in vitro* findings have recently been substantiated by *in vivo* injection of an avian leucosis retrovirus containing the alkaline phosphatase (AP) gene into the subventricular zone of transgenic mice. These mice express the avian leucosis receptor under the control of the glial fibrillary acidic protein (GFAP) promoter. Thus, the retrovirus label only astrocytes with AP. Yet, 14 days later AP positive interneurons were found in the olfactory bulb (31). From these data, it seems that within certain brain regions there are cells that are capable of transdifferentiating between several neural cell lineages giving rise to both neurons and glia. It is still an open question if it is single pluripotent neural cells within the brain that give rise to gliomas. However, it is clear that glioma cells can express markers of neural precursor cells, and their pluripotent nature may well explain the cellular origin of mixed gliomas, i.e gliomas expressing different glial phenotypes.

3.2. Glioma cells can express neural precursor phenotypes

Neural stem cells transplanted into the adult brain show extensive infiltration within the CNS, a trait which is also shared by malignant gliomas. This raises the question whether stem cells give rise to gliomas.

It has been shown that glioma cells may express a variety of antigens expressed by developing neural cells, e.g. the intermediate filament proteins nestin and vimentin (32, 33), the NG2 proteoglycan and specific gangliosides (see below). Biochemical analyses of autopsy brains from individuals diagnosed with gliomas have shown that brain areas invaded by tumor cells contain relatively large amounts of the gangliosides 3'-isoLM1 and 3´6´-isolLD1 (34). These gangliosides are not expressed in the normal adult brain (after two years of age), but are found during brain development in association with glial proliferation and migration, with the highest peak (10nmol sialic acid/g tissue) during the first trimester (35, 36, 37). This may imply that 3'-isoLM1 has a function during neural as well as glioma cell migration.

NG2 is known to be expressed during embryogenesis as early as embryonic day 12, and is especially associated with brain capillaries (38). NG2 is expressed throughout the period of rapid expansion of the brain vasculature and is down-regulated as the vessels

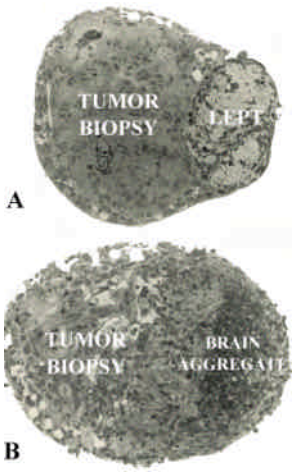

Figure 1. A: Co-culture between a leptomeningeal aggregate (LEPT) and a tumor spheroid derived from a glioblastoma biopsy. No invasion into the leptomeningeal tissue is observed (arrows; 72hr of confrontation (magnification x 300). B: The same tumor spheroids show an extensive diffuse invasion into rat brain cell aggregates reflecting the invasive behavior of the tumor *in situ* (magnification x 300). For further details see (48).

terminally differentiate (39). In the adult CNS, oligodendroglial precursor cells also express NG2 (40,41,42), but the molecule is also found to be expressed on glioma cells as well on neovasculature, in particular associated with pericytes (43).

We have recently shown, by overexpressing NG2 in human U-251 glioma cells, that this leads to an increase in tumor initiation and intracerebral growth in nude rats. Immunohistochemical analyses and fMRI showed that tumours where highly vascularized and had an substantial cell proliferation which predisposed to a poorer survival outcome (43). Furthermore we have shown that NG2 may inactivate angiostatin and this may in part explain the hypervascularization of the tumours (43).

It is tempting to speculate that the migration of neural precursor cells actually represents the normal counterpart of glioma cell migration. The migratory behavior of gliomas may be due to a predetermined interplay between normal brain tissue and the migrating cells, where the brain represents a permissive tissue for guiding cells with certain phenotypic traits to migrate along specific anatomical structures.

In this context, it should be emphasized that the presence of multipotent cells in specific brain regions (like the subventricular zone) could potentially explain the distribution and differentiation capacity of a plethora of brain tumours. For instance, it has been shown that transplacental injection of ethylnitrosourea (ENU) or the intracerebral exposure to oncogenic viruses induces a high frequency of periventricular gliomas, often multifocal and of mixed cellular composition, as well as the generation of multipotent cerebellar medulloblastomas. Thus, the main loci of continuing post-natal neurogenesis may also be the sources of highly malignant and invasive tumours as astrocytomas, glioblastomas and cerebellar medulloblastomas (44). It should be emphasized that precursors found along the rostral-caudal axis may give rise to progenitors with distinct differentiation capacities (45). However, it is unclear to what extent this correlate with the distribution and differentiation of gliomas.

4. MECHANISMS OF TUMOR CELL INVASION

The mechanisms that determine the spread of glioma cells inside the CNS are extremely complex but can be divided into three distinct biological entities involving both biophysical as well as biological parameters. These include anatomical barriers, passive cell displacement by the flow of the cerebrospinal fluid and active cell movement.

4.1. Anatomical barriers

Metastases from primary brain tumours outside the CNS are extremely rare. A substantial number of extraneural tumour types can, however, metastasize to the CNS, which suggests that there are specific biochemical and anatomical barriers that prevent the gliomas from metastasizing out of the CNS. That gliomas can grow at extraneural sites, was shown by Bloom et al. four decades ago (46). In an attempt to stimulate an immune response, small GBM biopsy samples were at the time of surgery, autografted subcutaneously to the thighs of the patients. Two patients died of their intracranial tumours, while the rest developed large tumours at the site of implantation (46).

It is well known that glioma cells frequently form cuffs around blood vessels within the perivascular space (47). The perivascular space is lined by the basal lamina associated with the endothelial cells, smooth muscle cells and projections of leptomeningeal cells, all of which are attractive candidates as barriers preventing extraneural metastases. In this context, we have shown that glioma biopsy spheroids are unable to invade cellular aggregates prepared from leptomeningeal cells, but show a diffuse invasion into brain cell aggregates *in vitro* (Figure 1). Moreover, the leptomeningeal cell aggregates were readily invaded by brain metastases from small cell lung carcinomas (48).

Even though the leptomeninges in many brain regions do not form a dense barrier, the study above indicates that cellular barriers exist which are permissive for extraneural metastatic tumours but not for gliomas (48).

Tumor cell extravasation depends on a penetration of the basal lamina. This event is probably not possible without a degradation of basement membrane components and a specific adhesion of circulating tumor cells to endothelial cells in a specific target organ. Besides physical barriers, it is also possible that the glioma cells lack specific endothelial cell surface attachment receptors. For instance, it has been shown that glioma cells *in vitro*, lack the cluster of differentiation 15 (CD 15) epitope,

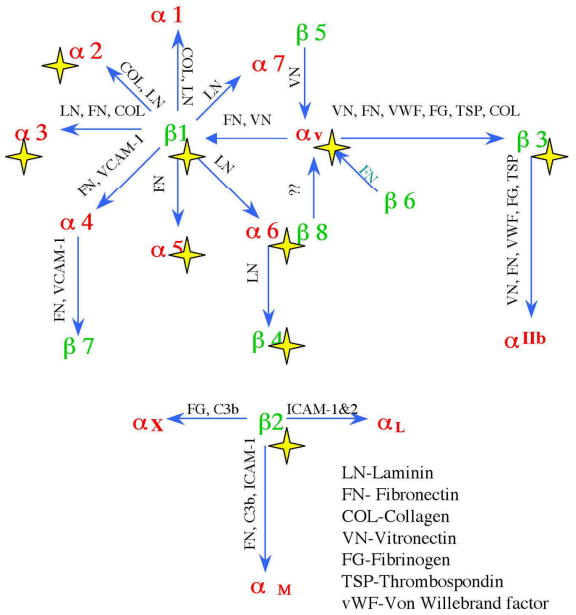

Figure 2. Illustration of the integrin system where different alpha-subunits can associate with a number of different beta units to form functional receptors that may interact with a variety of ECM molecules. The stars depict the integrin subunits that have been reported to be involved in glioma cell invasion.

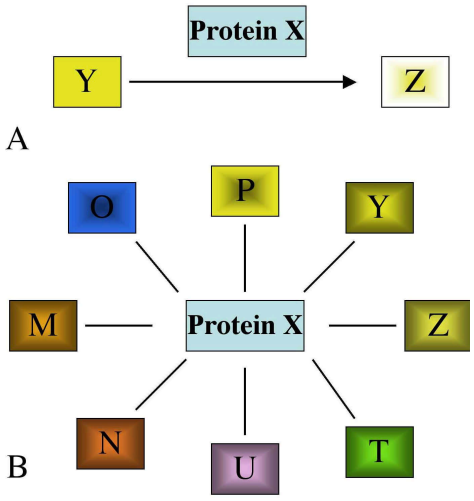

Figure 3. A: The classical and somewhat simple way of looking at protein function, i.e. protein X acts on protein Y to form Z. B: The post genomic network view of protein function is that the function of protein X is in the context of its interaction with several proteins (adapted from Eisenberg).

which recognizes cell adhesion molecules on endothelial cells (49).

4.2. Passive cell displacement

Transplantation studies of tumor cells as well as inert fluorescent latex particles within the CNS, have shown that both fluorescent particles as well as tumor cells may be extensively distributed within the CSF compartments (50). The extensive production (500ml/day) and the high absorption of CSF in a relative small volume (150ml) imply a substantial CSF flow within the brain. The most attractive explanation for the widespread distribution of particles and cells in the perivascular and subarachnoid spaces may be a passive transportation along the CSF flow. However, to what extent the passive flow contribute to the actual spread of tumor cells within the CNS has not yet been clarified.

4.3. Active cell movement

Active cell movement implies an activation of numerous intra- as well as extracellular signaling cues. This involves specific cell surface receptors interacting with numerous extracellular matrix molecules (ECM), the secretion of numerous proteases, cell signaling events as well as a functional cytoskeleton. In the search for mechanisms for tumor cell invasion, numerous "reductionistic" approaches have been undertaken to identify key molecules or intracellular signaling pathways responsible for the invasive process. By performing a Medline search, at least 22 different ECM components have been identified to be involved in glioma cell invasion. Interacting with these are numerous cell surface receptors and proteolytic enzymes. Cell surface receptors that have been closely associated with glioma cell invasion are the integrins. The integrins are a group of heterodimeric cell surface glycoproteins which are composed of two subunits referred to as alpha?and beta chains. At least 15 different alpha-subunits can associate with at least 8 different beta units to form functional receptors (51). It is mainly through the interactions of these molecules with a variety of ECM molecules that cells are able to attach, differentiate, spread and migrate within the organism (52). Several different integrin dimers have been shown to be implicated in glioma cell invasion. This underscores the complexity of the cell signaling events that take place during the invasive process (Figure 2). Similarly a substantial number of proteases have also been shown to be implicated in glioma cell invasion, among which the serine proteases and metalloproteases seem to play an important role (53, 54).

In recent years, analyses of global genomic and protein expression profiles have started to elucidate molecular portraits that may define the malignant phenotype. Such portraits are based on complex intra as well as extracellular molecular communication networks within and between cells. The classical way of looking at protein function focuses on the action of a single molecule. This action may be the catalysis of a given reaction influencing another molecule. In the expanded view of protein function, a protein may be defined as an element in the network of its interactions (Figure 3).

At present new methods are being devised to obtain functional information for many proteins at once. It is expected that assignments can be made to a large number of proteins encoded by the genome. Such methods often detect the functional linkage between proteins. If one protein is known it can be inferred that the linked proteins act in the same pathway or network as the first protein (55). A powerful method is the two-hybrid screen to genome

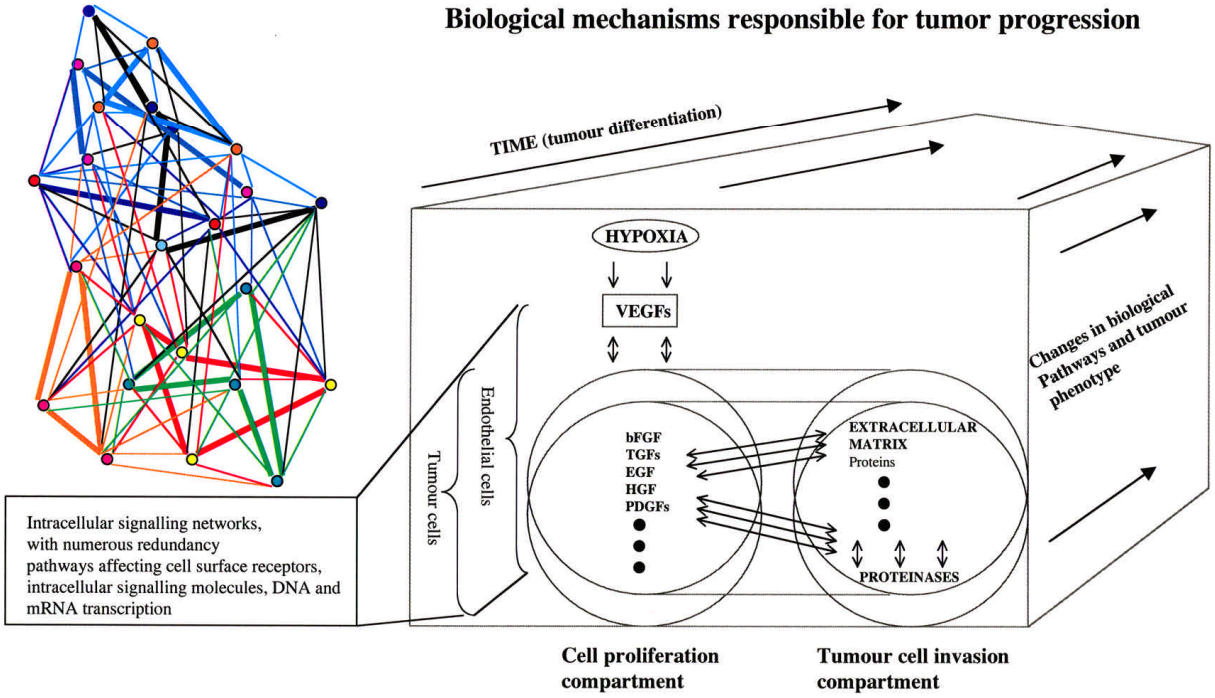

Figure 4. Schematic presentation of major cell communication networks within cells as well as between tumour and endothelial cells. Tumour progression involves two closely linked cell signaling events involving cytokines that regulate cell proliferation and proteinases that may affect tumor cell invasion. In order for cell proliferation to take place, several cell signaling cues mediated by specific growth factors are shared between the glioma cells and the endothelial cells while others, as for instance the vascular endothelial growth factors (VEGFs), are unique for the endothelial cells. Numerous growth factors may induce proteinase production that eventually may lead to breakdown of ECM components followed by local tumour and endothelial cell invasion. It is well known that the ECM represents a reservoir for growth factors which in turn may induce cell proliferation. Such joint communication networks (interactions between cytokines, proteinases and ECM) may change as the tumour progress.

wide assay which has identified thousands of putative protein-protein interactions in yeast cells (56). By the rapid advancement of functional genomics a major challenge will be to understand the complex cell signaling events that take place during tumor development. After working with numerous cell surface receptors and ECM components over the years, it is our experience that the gliomas have a tendency to adapt to several ECM substrates, and that they are quite opportunistic in this respect (57). This indicates that a heterogeneous tumour cell population may use numerous inter- as well as intracellular signaling events that eventually will lead to active cell movement within the brain. Most likely, this behavior reflects the plasticity of the human genome and the numerous proteins that provide the functional information within and between cells. In order to understand glioma cell invasion, identified proteins responsible for the invasive process should be defined as an element in the network of its interactions, where it is the network rather than a specific molecule that defines the invasive phenotype (Figure 4). The progression of gliomas seems to involve two closely linked cell-signaling events; those involved in cell proliferation and those involved in tumor cell invasion. Two major cell types are involved, i.e. the tumor cells and the endothelial cells. In order for cell proliferation to take place, several cell signaling cues mediated by specific growth factors are shared between the glioma cells and the endothelial cells while others, as for instance the vascular endothelial growth factors (VEGFs), are unique for the endothelial cells. Similarly, joint communication networks (interactions with ECM components and protease secretion) may also be shared between the endothelial cells and the tumour cells (Figure 4).

In conclusion, to understand the underlying mechanisms for the progression of gliomas, it will be important to identify major cell communication networks and possible redundancy in the networks within the tumours. In order to perform such complex tasks, the scientific community has to rely on the rapid advancements within the field of genomics, proteomics and bio-informatics. Furthermore, in order to get a comprehensive understanding of the disease, experimental animal models have to be developed that reliably reflect the malignant progression of human tumours *in vivo.*

5. THE ANGIOGENIC PROCESS

Angiogenesis is defined as the sprouting of new vascular endothelial cells (EC) from pre-existing capillaries. The turnover of EC in normal tissues is less than 0.01%, indicating replacement of single EC rather than active angiogenesis (58, 59). During some physiological

Table 1. Endogenous angiogenesis inhibitors

Inhibitors	Fragments	Parent protein/tissue origin	Reference
Specific inhibitors of EC			
▪ Angiostatin	x	Plasminogen	79
▪ Endostatin	x	Collagen XVIII	80
▪ Kringle 5	x	Plasminogen	81
▪ Kringle 1-5	x	Plasminogen	82
▪ Vasostatin	x	Calreticulin	83
▪ Serpin antithrombin		Antithrombin	84
Cytokines			
▪ TGF-β1			85
▪ PF-4			86
▪ IL-12			87
▪ IL-18			82
▪ INF-α			88
▪ INF-γ			87
▪ Chemokine gro-β			89
▪ Chemokine IP-10			90
Antagonists of angiogenic factors			
▪ Soluble VEGFR-1			91
▪ Soluble FGFR-1			92
▪ Angiopoetin-2			93
Matrix-protein related inhibitors			
▪ TSP-1			94
▪ METH-1, 2			95
▪ TIMPs			96
▪ PEX	x	MMP-2	97
▪ Maspin			98
▪ Canstatin		Collagen IV	99
▪ Arresten		Collagen IV	100
▪ Tumstatin		Collagen IV	101
Derived from avascular tissues			
▪ PEDF		Retinal cells	102
▪ Troponin-1		Cartilages	103
Miscellaneous inhibitors			
▪ 16-kD prolactin	x	Prolactin	104
▪ PRP		Placenta	105
▪ Restin		Collagen XV	106

TGF = transforming growth factor; PF = platelet growth factor; IL = interleukin; INF = interferon; IP-10 = inducible protein 10; TPS = thrombospondin; METH = metalloproteinase-thrombospondin; TIMP = tissue inhibitors of metalloproteinases; PRP = proliferin-related protein.

conditions such as in muscle exercise, tissue repair, the female reproductive cycle and embryogenesis, angiogenesis is switched on. Physiological angiogenesis is of limited duration and tightly regulated. Pathological angiogenesis occurs in a variety of diseases such as rheumatoid arthritis, retinal neovascularization and growth and progression of benign and malignant tumours including gliomas (60).

5.1. Angiogenic inducers and inhibitors

In recent years, numerous angiogenesis inducers and angiogenesis inhibitors, endogenous as well as synthetic, have been identified (Table 1) and their role in normal and pathological conditions has been investigated (61, 62). Tumor angiogenesis is a complex process involving the release of angiogenic factors such as basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) (step I), proteolytic degradation of ECM which also serves as a reservoir for angiogenic and anti-angiogenic factors (step II), proliferation of EC (step III), directional migration, composition of new vessels and stabilization of the immature vessels by pericytes (step IV). bFGF has been shown to promote invasion of human glioma cell lines into brain cell aggregates *in vitro*, i.e in a system devoid of angiogenesis. At the same time it has been shown, by expressing dominant negative FGFR1 or FGFR2 on C6 glioma cells, that inhibition of bFGF activity may disrupt angiogenesis-dependent and independent signals required for glioma growth and invasion. (63,64). The change from quiescent vasculature to the angiogenic phenotype is believed to result from an angiogenic switch mechanism, where the balance between inducers and inhibitors of angiogenesis is displaced (65). VEGF is the key inducer of angiogenesis in both physiological and pathological conditions, and is produced by various cells although its receptors (Flt-1, Flk-1 and Flt-4) are restricted to EC (66, 67). VEGF also induces vascular permeability (68). Its expression depends on activated oncogenes/inactivated tumor suppressor genes, and is strongly

up-regulated under hypoxic conditions (69). Angiostatin, endostatin and thrombospondin-1 (TSP-1) have been identified as potent inhibitors of angiogenesis. Such inhibitors are stored and can be produced locally by proteolytic cleavage of a larger protein which is either soluble or part of the ECM (65).

A diagnostic hallmark of GBM is microvascular proliferation (MVP). MVP is typically florid, making GBM one of the most highly vascularized human neoplasm and an attractive candidate for antiangiogenic therapy (70).

The vasculature in GBM is characterized by multilayered swollen EC, permeable vessels and local thrombosis (71). Frequently, leaky blood vessels lead to capillary effusion and peritumoral oedema. The most prominent form of MVP is called "glomeruloid" MVP because of the formation of coiled, glomerulus-like capillary loops (72), which frequently are associated with GBM. VEGF is up-regulated during glioma progression, and is likely to facilitate both angiogenesis and vascular permeability, a notion that is supported by down-regulation of VEGF-expression after treatment with dexamethasone (73). VEGFs seem to be induced in GMBs as a response to hypoxia (74).

Glioblastomas show an extensive infiltration of the brain parenchyma (75). Experimental reports do causally link glioma cell invasion to angiogenesis. As before mentioned, bFGF stimulates both migration and angiogenesis in gliomas (63, 76). Furthermore, supporting histopathological observations and intravital microscopy has shown that C6 glioma xenografts migrate along blood vessels *in vivo* (77). When targeting angiogenesis with SU5416, which is a selective inhibitor of the VEGF/Flk-1 signal transduction pathway (Table 2), the expression of pro-invasive proteins is down-regulated and glioma cell infiltration is inhibited (78).

It is well known that GBM frequently show loss of tumor suppressor genes on chromosome 10. Interestingly, the insertion of wild type chromosome 10 in human glioma cell lines may lead to a reduced ability to form tumours in nude mice, by switching to a non-angiogenic phenotype. Such a change has been attributed to the secretion of TSP-1 (107) or by PTEN activation which has also been associated with angiogenesis regulation (108). Furthermore, it has been shown that the introduction of anoxia will reduce the expression of TSP-1 in glioma cells. This implies that reduced oxygen tension can promote angiogenesis, not only by stimulating inducers like VEGF, but also by reduction of inhibitors (109). Besides affecting endothelial cells TSP-1 may also regulate or direct the cytoskeletal organization of glioma cells. This process can be modulated by retinoic acid (110).

Angiopoietin-1 (Ang-1) and its naturally occurring antagonist angiopoietin-2 (Ang-2) are ligands that regulate tyrosine phosphorylation of the Tie2/Tek receptors on endothelial cells. It has been shown that angiopoietins are involved both during the early stages of vascular activation as well as at later stages of advanced angiogenesis. Especially Ang-2 has been identified as an early marker of glioma induced neo-vascularization where it may play a role in tumor associated activation of pericytes and smooth muscle cells. For example all microvessels in gliomas, which display dynamic changes in alpha-smooth muscle actin, also express Ang-2 mRNA (111).

The local invasion of blood vessels in gliomas is in part mediated by integrin-dependent cell adhesion mechanisms where especially the alphaVbeta3 and alphaVbeta5 seem to be important for vascular sprouting (112). Other factors that have been directly or indirectly associated with glioma angiogenesis are epidermal growth factor (EGF) and scatter factor (hepatocyte growth factor) (113).

6. NEW THERAPEUTIC STRATEGIES FOR GLIOMAS

Several angiostatic proteins have a relative short biological half-life in vivo. Gene therapy may therefore represent a valid approach for the delivery of such proteins to the host. By the use of retroviral and adenoviral vectors, a number of angiostatic proteins have been delivered to experimental tumours in vivo (114). This includes the in situ delivery of retroviral vectors expressing IL-4 (115), dominant negativeVEGFR-2 (116), INFγ (117) and of Semiliki Forest virus carrying endostatin (118). All these experiments resulted in reduced tumor growth.

Although gene therapy theoretically offers a promising new treatment modality, it has to date failed to show its definite role in cancer therapy. The field still faces problems of gene delivery, selectivity and efficacy. Initiatives should focus on these aspects, as well as on alternative systems for the delivery of angiostatic molecules to tumours.

6.1. Antiangiogenic therapy

Antiangiogenic therapy may seem appealing for several reasons. First, it allows selective targeting based on the phenotypically distinct differences between immature and quiescent vessels, and since physiological angiogenesis is turned off in adults, antiangiogenic therapy should imply minimal side effects (119,120,121,122). Furthermore, tumor vessel EC should not induce drug resistance, which is a major problem in cancer therapy (123). Since the angiogenesis inhibitors are distributed within the vascular system, the drugs are easily distributed to the EC of the tumor vasculature. Last but not least, antiangiogenic compounds may act synergistically with conventional therapeutic regimens. Concerns do however exist regarding side effects on tissue repair processes, the reproductive cycle and potential teratogenic effects. Thalidomide is an example of an antiangiogenic agent that is highly teratogenic because of its inhibition of EC growth and vessel development in human embryos.

Numerous clinical trials have been initiated for a variety of malignancies, using synthetic as well as naturally occurring antiangiogenic compounds. The substances affect angiogenesis at different levels, being inhibitors that directly affect EC or indirectly through depletion of copper, inhibitors of matrix metalloproteinases (MMPI), antagonists

Table 2. Angiogenesis inhibitors in clinical trials [1]

Therapeutic substance	Phase	Astrocytomas	
Endothelial cell inhibitors			
▪ Endostatin	II		
▪ Angiostatin	I		
▪ TNP-470	II	X	MT
▪ Squalamine	I/II	X	MT
▪ Thalidomide	II	X	MT; +CT
▪ ME-2	II		
▪ Combretastatin	I		
▪ FTI	I	X	
▪ CM101/ZDO101	I		
Chelators of copper			
▪ Penicillamine	II	X	Copper-low diet
▪ Tetrathiomolybdate	I/II		
▪ Captopril	I/II		
▪ MMPI			
▪ Marimastat BB-2516	III	X	MT; + γ-knife
▪ Neovastat Æ941	III		
▪ Prinomastat AG3340	III	X	+ CT
▪ BAY 12-9566	III		
▪ COL-3	I	X	MT
▪ BMS-275291	I		
▪ CGS 27023A	I/II		
VEGF-antagonists			
▪ Anti-VEGF antibody	III		
▪ PTK787/ZK22584	I/II		
▪ SU-5416	I	X	MT
▪ SU-66668	I		
Ribozyme Angiozyme	**I**		
▪ Cytokines			
▪ INF-2α	II/III		
▪ rPF4	I/II	X	MT
▪ IL-12	I		
Integrin-inhibitors			
▪ EMD121974	I/II	X	MT
▪ Vitaxin	II		
▪ Others			
▪ CAI	II	X	+ CT
▪ IM862	III		
▪ Celecoxib	II		
▪ Suramin	II	X	MT
▪ PNU-145156E	I		
▪ ABT-627	I	X	

FTI= farnesyl-transferase inhibitor; MMPI= matrix metalloproteinase inhibitors; INF=interferon; PF4=platelet factor 4; IL= interleukin. MT= monotherapy; + CT= combined with chemotherapeutic agents. [1] According to the registration of NCI Cancer Trials, EntreMed Inc., www.cancer.gov and www.entremed.com and (141).

of the VEGF signal transduction pathways, antiangiogenic cytokines, inhibitors of the vascular integrin $\alpha_V\beta_3$, and other compounds that are not easily categorized (Table 2). Current reports from phase I and II clinical trials, conclude that the angiogenesis-inhibitors are well tolerated (124, 125). For the endogenous inhibitors endostatin and angiostatin, no dose limiting toxicity has been reported (126, 127). Results from only a few phase II trials have been published, indicating none or limited therapeutic effects on patients suffering from advanced therapy-resistant malignancies. Combination regimens with e.g. cytotoxic agents have therefore been suggested (128, 129, 130, 131).

GBM are as afore-mentioned highly vascularized. Even in low grade astrocytomas, VEGF expression and vascular density are prognostic markers of malignant progression (132). Experiments in animals using anti-VEGF antibodies (133), VEGF-receptor mutants (134), antisense

Table 3. Selected experimental protocols applying cell encapsulation technology.

Disease	Device	Outcome	Reference
Diabetes	Alginate mic with Langerhans Islets	Normoglycemia of canines for 125 days after i.p. injection of mic, one dog was normoglycemic for >2yrs.	145
Liver failure	Macrocapsular devices	Several devices exist and are regarded to be sufficiently successful to enable patients to survive the waiting period for transplantation.	145
Kidney failure	Alginate mic with E.coli DH5α	Given orally for 21 days to uremic rats resulted in lowered and maintained urea levels at the normal range. Mic left the animals with the stool.	147
Hypopara-Thyroidism	Alginate mic with human parathyroid tissue	Normocalcemia lasting>6 months in rats.	148
Dwarfism	Alginate mic with recombinant cells expressing hGH	Correction of dwarfism in mice, high viability maintained >6 months. hGH was secreted for >1month after s.c. implantation in canines.	149 150
Chronic pain	Alginate mic with chromaffine cells	Successful implantation for 4-8 weeks of xenograft mic in the subarachnoid space of large animals.	151
Haemophilia	Alginate mic with recombinant cells expressing human factor IX	Factor IX was secreted >213 days postimplantation into mice. However, human antibodies to factor IX accelerated the clearance of factor IX.	152
Lysosmal storage disease	β-glucoronidase	Intraventricular implantation into mice CNS leads to reduction in lysosomal storage lesions near the implantation site and normalized behaviour.	153

mic=microcapsules; i.p.=intraperitoneal; s.c.= subcutaneous; PTH=parathyroid hormone; hGH=human growth hormone; EPO=erythropoietin; CNTF=ciliary-derived neurotrophic factor

VEGF (135) and the VEGFR-2 (Flk-1)-inhibitor SU-5416 (136) have all showed a marked inhibition of anigogenesis, invasion and tumor growth in vivo.

Numerous other angiogenesis inhibitors, such as endogenous angiostatin (137) and endostatin (138), TNP-470 (139) and suramin (140), have been shown to effectively target the neovasculature in experimental gliomas (141). Several of these agents are in clinical trials, either as monotherapy or in combination with cytotoxic agents (Table 2). The results of these trials are pending.

6.2. Cell-based delivery of therapeutic substances

Cell-based delivery systems for biologic compounds with a therapeutic potential represents a new therapeutic approach, which utilizes ex vivo transduction of a universal cell line with a therapeutic gene (142,143,144). The recombinant cells which are generated express and secrete the therapeutic protein, and may be implanted in vivo directly or upon encapsulation in immunoisolating substances, thus forming "factories" or "bioreactors" for protein-production. Allografted recombinant cells may have a limited lifespan in the host due to immunological rejection. Encapsulation devices represent a way to circumvent immune activation and still achieve local sustained delivery of bioactive compounds. The concept of using encapsulation for immunoprotection of transplanted cells has been used in a variety of experimental protocols, mainly as hormone substitution or for transplantation purposes of bioartificial organs. To illustrate the general applicability of cell encapsulation, some experimental trials and their outcome are listed in Table 3.

The use of alginate microcapsules in the CNS is believed to be particularly beneficial as compared to implantations at other sites based on the privileged immunological environment in the brain. Alginates consist of naturally occurring, un-branched anionic polysaccharides that consist of alpha-L-guluronic acid (G) and beta-D-mannuronic acid (M). G and M are monosaccharides that are arranged as alternating chains that may vary in complexity. In the presence of multivalent cations (e.g Ca^{2+}, Ba^{2+}, Fe^{2+}) the alginate will form a gel matrix. Most of the current transplantation work is done with Ca^{2+} and Ba^{2+} alginates (154).

Recently, we and others have demonstrated a pronounced inhibition of rodent brain tumours by the implantation of alginate bioreactors producing endostatin (143, 144). A major benefit of implanting encapsulated protein-expressing cells is the possibility of sustained delivery of an active protein at near physiological concentrations. Furthermore, we have shown that the brain tolerates alginate implants well with little immunoreactivity (155,156,157). Many protein-based therapies are hampered by unfavorable pharmacokinetic profiles and in this respect cell-based delivery systems should have a considerable potential.

7. FUTURE PROSPECTS

It is well known that most tumours are of monoclonal origin, i.e. derived from one single transformed cell (158). This cell and the cell populations that subsequently evolve are highly adapted to the host microenvironment. Thus, the tumor developing tumour avoids immune surveillance mechanisms and shows a striking flexibility in using specific genes or genetic programs that are tailored to the microenvironment in which it grows. With the advancement of differential display techniques to study variations in gene expression

and protein expression, several gene and protein communication networks that play important roles during tumor progression, will be identified in following years. To further characterize the vast number of genes that in fact regulate tumour growth and progression, gene array and Serial Analysis of Gene Expression (SAGE) technologies represent interesting novel approaches (159). Using SAGE, several thousand cellular transcripts can be identified by numerous base pair tags (10 bp pr tag). SAGE has been used to compare gene expression in human glioblastomas. Approximately 56000 unique transcripts were analysed. In comparison to normal CNS white matter, 471 transcripts were increased or decreased more than five fold in human GBM. A functional classification of the differentially expressed genes has revealed angiogenesis related genes, transcription factors, and cell cycle regulators, providing a pool of candidate genes involved in glioblastoma pathogenesis. These data are available at http://www.ncbi.nlm.nih.gov/SAGE as part of the Cancer Genome Anatomy Project (CGAP).

The whole human genome is now sequenced. With the advancement of micro-robotics, it is possible to print up to 45 000 known cDNA sequences as one array on a cover glass (DNA chip) whereupon cDNA from a tumour as well as normal tissue can be hybridized. By this approach, utilizing special scanners and computers, the research environment is now characterizing specific gene clusters that are up or down-regulated and which account for a specific malignant phenotype. By using microarrays it should also be possible to characterize in detail the different gene portraits that regulate the invasive phenotype of malignant brain tumours and also to determine to what extent such portraits may be affected by new therapeutic strategies.

For instance genetic determinants of glioma invasion *in vivo* has been identified by comparing mRNA array expression profiles of GBM cells residing within the tumor core and those obtained at the invasive rim of resected human tumours. By using this approach apoptosis resistance genes as well as genes associated with cell migration have been described (160). Thus, the microarrays should provide new insight into how specific gene clusters are related to diseased tissue, and this technology may therefore represent a new diagnostic tool for malignant diseases including tumours of the CNS.

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Abbreviations: bFGF-basic fibroblast growth factor, TGF-transforming growth factor, EGF-epidermal cell growth factor, HGF-hepatocyte growth factor, PDGF-platelet derived growth factor

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