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# The Blood-Brain Barrier in Brain Tumours

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## 1. Introduction

The literature on glioblastoma is increasingly concerned with genetic pathways to and genomic analyses of glioblastoma (Ohgaki et al., 2004; Parsons et al., 2008). Mutations of numerous genes were demonstrated to be involved in the development of brain tumours; however, in most cases the mechanistic roles of these mutated genes in the dysregulation of the cells of tumour origin are not understood in functional terms, but are used for molecular diagnostics (Riemenschneider et al., 2010). Moreover, several potential biomarkers of glioblastoma were identified and classified for clinical prognosis without precise knowledge of their function (Sreekanthreddy et al., 2010). A new dimension of brain tumour research has been reached by the detection of cancer stem cells in glioblastoma which were identified as a small subpopulation of brain tumour propagating cells (Huang et al., 2010; Tabatabai & Weller, 2011). A link between glioblastoma stem cells and tumour vascularization has been established by the first description of the possibility that tumour vessels can be recruited from glioblastoma stem cells (Ricci-Vitiani et al., 2010). This opens a new field concerning the plasticity of tumour vessel endothelial cells including their lost or undifferentiated barrier properties. Since the clinical signs of the glioblastoma are connected to the intracerebral pressure due to edemas, understanding the alterations of the blood-brain barrier (BBB) is of central importance. For this reason, we will begin this chapter on the blood-tumour barrier with the description of cell biological aspects of the healthy BBB.

The BBB is responsible for the homeostasis of the microenvironment in the neural parenchyma and essential for normal function of the brain. In the strict sense, it is located in the endothelial cells and restricts the paracellular diffusion of hydrophilic molecules by both complex tight junctions (Reese & Karnovsky, 1967; Brightman & Reese, 1969) and a low degree of transcytosis (Peters et al., 1991). This implies the necessity of various specific transporters for providing the brain with compounds essential for brain energy metabolism (Begley, 2004).

It is generally accepted now that astrocytes play a decisive role in the maintenance if not induction of the BBB (Abbott et al., 2006; Wolburg et al., 2009). But we have to be aware that the mechanism by which this maintenance or induction of the BBB is performed, is not understood so far. In any case, in the mature brain, the astrocytes embrace the vessels by sending an endfoot towards the perivascular basal lamina (Mathiisen et al., 2010). It has

been well-known for many years that the astroglial membranes contacting the subendothelial or pericytic basal lamina are characterized by the occurrence of orthogonal arrays of particles (OAPs). These arrays can, up to now, exclusively be demonstrated by the freeze-fracturing technique (for a recent survey, see Wolburg et al., 2011), but it is known that they consist of the water channel protein aquaporin-4 (AQP4). Where the glial membrane loses the contact with the basal lamina by diving into the neuropil for making contacts with other astroglial cells, oligodendrocytes, neurons and synapses, the density of OAPs drops dramatically. The distribution of OAPs in both astroglial membrane domains, the perivascular endfoot membrane and the non-endfoot membrane, can be described as a polarization of the astrocyte. Interestingly, this polarity of astrocytes arises concomitantly with the maturation of the BBB, and is not maintained by cultured astrocytes (Nico et al., 2001). In the context of this chapter, it is important that the polarity of glioma cells heavily decreases (Neuhaus, 1990; Noell et al., submitted). It will take some space in this review to describe the circumstances under which the polarity is decreased and which consequences will arise for the pathophysiology of brain tumours.

## **2. The healthy blood-brain barrier**

### **2.1 Endothelial cells**

The endothelial cells of the BBB have been shown to form the most complex tight junction networks among all endothelial cells of the entire vasculature of the body (Nagy et al., 1984). Previously, the complexity of the network of tight junction strands has been used for the prediction of the physiological parameters, permeability and transepithelial electrical resistance (Claude, 1978). However, this relationship between morphological and physiological parameters has originally been established for epithelial cells: its validity for endothelial cells was confirmed on the basis of Claude's paradigm. In the last years, many details of the molecular composition of tight junctions have been published. Most of these results have been obtained from studies on epithelial cells, and only few publications have tried to compare molecular aspects of endothelial, and in particular BBB endothelial, tight junctions with those of epithelial cells (Wolburg & Lippoldt, 2002). ZO-1 was the first protein identified and characterized as a tight junction-associated protein (Stevenson et al., 1986). ZO-1 is a cytoplasmic 220 kDa phosphoprotein of the membrane associated guanylate kinase homologues (MAGUK) family. The localization of ZO-1 to the tight junction is not exclusive (Itoh et al., 1993), as in cellular systems with less elaborate or no tight junctions, ZO-1 is found enriched in regions of the adherens or gap junctions (Giepmans & Moolenaar, 1998; Itoh et al., 1993). ZO-2, a 160kDa protein of the same MAGUK family, turned out to be a ubiquitous component of epithelial and endothelial tight junctions (Jesaitis & Goodenough, 1994). Independently of ZO-1, ZO-2 can determine claudin polymerization in the tight junction strands (Umeda et al., 2006). The family of claudins turned out to be the most important molecules of tight junctions, because they are the permeability-restricting molecules proper (Furuse et al., 1998; Morita et al., 1999a). Prior to the discovery of claudins, occludin was detected by the Tsukita group as well (Furuse et al., 1993). Both occludin and the claudins are membrane proteins with four transmembrane domains, which are nevertheless not homologue. Initially, occludin has been assumed to be essential for tight junction integrity, but the occludin-knock out mouse is viable and has no essential morphological phenotype and normally structured tight junctions in all organs (Saitou et al., 2000). Accordingly, occludin is presently believed to act in a yet undefined regulatory

context rather than as a major structural tight junction protein. The same is true for the recently detected tight junction molecule MarvelD3 which has been described as a new occludin family member not essential for the formation of functional tight junctions but determining paracellular permeability (Steed et al., 2009). In addition to the four-span transmembrane proteins, another family of single-span proteins occur at tight junctions, the junctional adhesion molecules (JAMs) including JAM-A,-B,-C, the coxsackie and adenovirus-associated receptor (CAR) and the endothelial selective adhesion molecule (ESAM) (Ebnet et al., 2004).

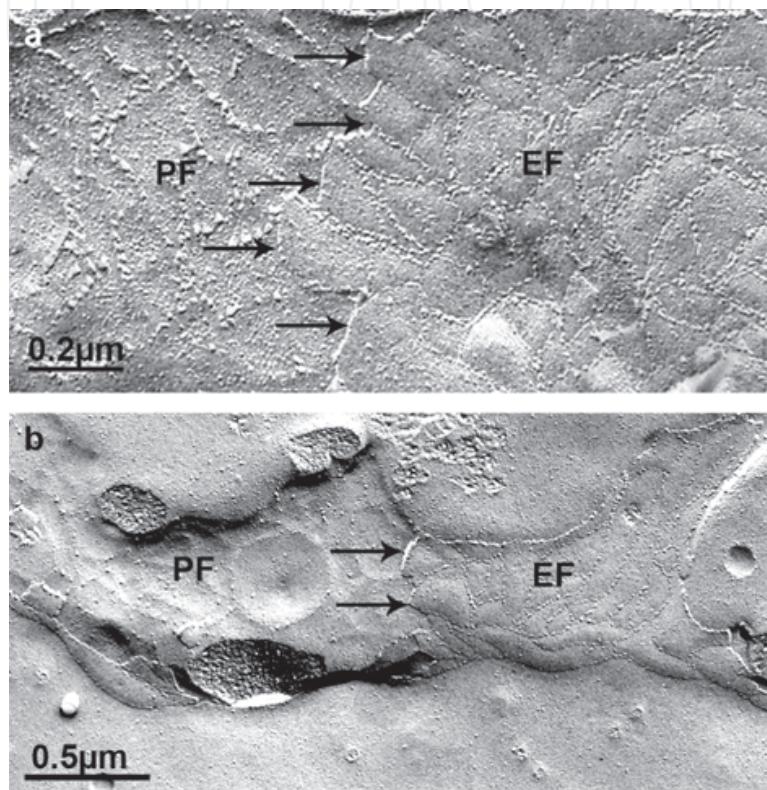


Fig. 1. Freeze-fracture replicas of endothelial cells in vivo (a) and in vitro (b). In vivo, BBB tight junctional strands have the highest degree of P-face (protoplasmic fracture face, PF) association found in the whole vasculature in the body. The most impressive alteration in vitro when compared with in vivo is the reduction of this association of the tight junctional strands with the PF. EF external fracture face or E-face. Arrows point to the switch from the P-face of the one cell to the E-face of the connected cell.

The molecular complexity of the tight junctions including the junctional scaffolding proteins such as ZO-1-3, cingulin, PAR-3, PAR-6, AF-6, MUPP1 or symplekin which are regulating the tight junctions are comprehensively described in a number of recent overviews (see, for example, Ebnet, 2008; Angelow et al., 2008; Krause et al., 2008; Balda & Matter, 2009; Steed et al., 2010).

In order to observe and describe tight junctions in terms of morphology, the freeze-fracture method still is most valuable (Fig. 1). The advantage of this method is that the two membranous lipidic bilayers are cleaved and shadowed with platinum/carbon vapour to replicate the molecular details of the fracture planes. Two aspects of the membrane can be distinguished: if the observer looks from outside the cell, the inner leaflet of the membrane is



displayed which is called the protoplasmic fracture face or P-face. If the observer looks from inside the cell, the outer leaflet of the membrane is displayed which is called the external fracture face or E-face. Concerning the tight junctions, two parameters can be visualized by freeze-fracture electron microscopy: the complexity of strands and the association of the particles with the P- or E-face. The complexity of the tight junction network was recognized to be related to the transepithelial electrical resistance (Claude, 1978). Nagy et al. (1984) investigated the tight junctions of the BBB and found them the most complex ones in the whole vasculature of the body. An additional parameter to describe the quality of the BBB tight junctions turned out to be the association of the tight junction particles with the P- or the E-face of the endothelial membrane (Wolburg et al., 1994). The BBB tight junctions are unique among all endothelial tight junctions in that their P-face association is as high as or even slightly higher than their E-face association (Fig. 1a). Interestingly, the P-face/E-face-ratio of BBB tight junctions continuously increases during development (Kniesel et al., 1996). In cell culture, the BBB endothelial cell tight junctions are mainly E-face-associated (Fig. 1b), and this is similar to non-BBB endothelial cells *in situ* (Wolburg et al., 1994) indirectly indicating that the association of the strand particles within the membrane leaflets is under the close control of the brain microenvironment. In our context, it is of particular interest that Claudin-1 and Claudin-3 led to the formation of tight junctions almost completely associated with the P-face when transfected into fibroblasts. In contrast, claudin-2 and claudin-5 transfected fibroblasts showed particles associated with the E-face (Tsukita & Furuse, 1999; Morita et al., 1999b). Therefore, the particle association with either the P- or E-face is believed to be a consequence of the stoichiometry of claudins within the tight junction. The dominant P-face-association is a particular feature of the BBB (Wolburg et al., 1994; Liebner et al., 2011).

The brain is faced with the dilemma between the necessary protection against neurotoxic compounds in the blood and against continuous variations of the blood composition on the one hand, and the delivery of energy-rich compounds to fuel the extremely demanding metabolism of the brain on the other hand. The second demand requires the presence of a lot of different transporters such as glucose transporters, amino acid transporters, anionic transporters or multidrug resistance transporters (for an overview, see Begley, 2004). The endothelial cells express a high density of glucose transporter molecules (which provides for glucose transport into the brain neuropil). The density of glucose transporters in the luminal membrane is three to four times lower than in the abluminal membrane (Farrell & Pardridge, 1991). Moreover, many Na<sup>+</sup>, K<sup>+</sup> pump molecules are localized asymmetrically in the abluminal membrane which facilitates the clearance of excess K<sup>+</sup> ions into the blood vessels, and is involved into the generation of osmotic forces for transendothelial water transport.

## 2.2 Astrocytes

The brain is the location where the interaction between vessels and surrounding tissue is extremely close. In no other organ, perhaps with the exception of the lung alveoles, endothelial cells are so intimately connected to non-endothelial cells. In the brain, these cells are the astrocytes which form so-called endfeet beneath the basal lamina around vessels and at the surface of the brain. Where the astrocyte processes touch the basal lamina, OAPs are inserted into the membrane: where the contact is lost, the OAP density drops sharply. The OAPs were described to contain the water channel protein aquaporin-4 (AQP4; Fig. 2; for reviews, see Rash et al., 2004; Rash, 2010; Wolburg et al., 2011). Aquaporins mediate water movements between the intracellular, interstitial, vascular and ventricular compartments which are under the strict control of osmotic and hydrostatic pressure gradients (Amiry-Moghaddam &

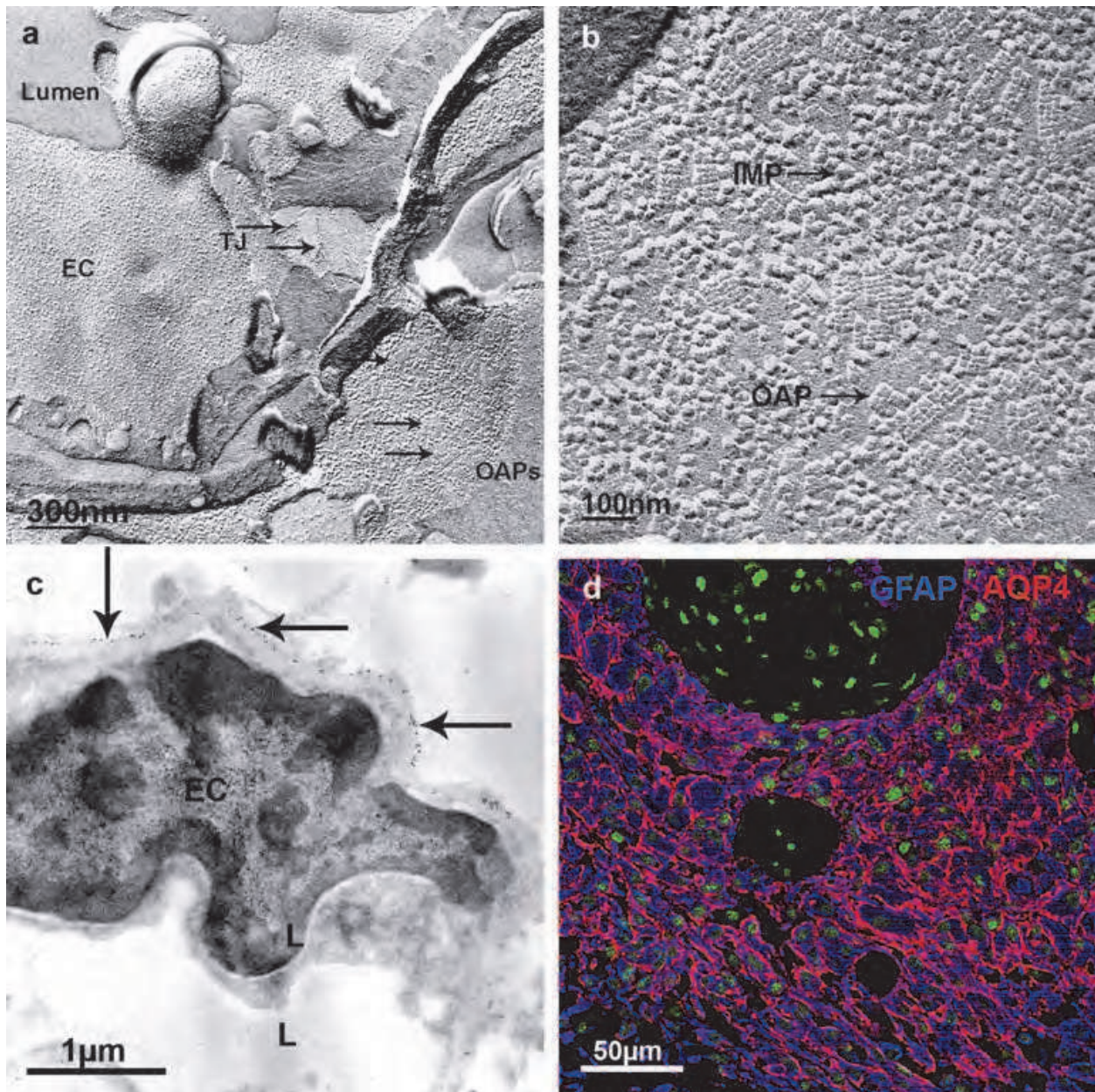


Fig. 2. Freeze-fracture replica (a,b; b is a detail of a) of the glio-vascular unit of the normal mouse brain, EC endothelial cell, TJ tight junction, OAPs orthogonal arrays of particles in the astroglial endfoot membrane, IMP intramembrane particles. The OAPs are located at and restricted to astroglial endfeet (from a collaboration with Christer Betsholtz, Karolinska Institute, Stockholm, Sweden). c: immunogold labeling of AQP4 in the astroglial membrane (arrows) around an endothelial cell (EC). L lumen of a microcapillary blood vessel. d: Immunohistochemical double labeling of AQP4 (red) and the glial fibrillary acidic protein (GFAP) in human glioblastoma. AQP4 is no longer restricted to glial (or glioma cell) endfeet, but distributed over the whole surface of the cell.

Ottersen, 2003; Benfenati & Ferroni, 2010; Pasantes-Morales & Cruz-Rangel, 2010; MacAulay & Zeuthen, 2010). The composition of the OAPs by AQP4 molecules is now generally accepted and was shown by several lines of evidence: the absence of OAPs in astrocytes of AQP4-deficient mice (Verbavatz et al., 1997), the formation of OAPs in chinese hamster ovary (CHO)



cells stably transfected with AQP4 (Yang et al., 1996), and the immunogold fracture-labeling technique directly showing that AQP4 is a component of the arrays (Rash et al., 1998). Moreover, Nielsen et al. (1997) were able to demonstrate by immunogold labeling that the distribution of the AQP4-related immunoreactivity in the retinal Müller glial cells was identical to that of the OAPs and was restricted to glial membranes.

Aquaporins in general form tetrameric protein complexes within the membrane plane (King et al., 2004). On the molecular level, each monomer represents a water channel proper (Tani et al., 2009). On the electron microscopical level, a structural subunit of the OAP measuring about 7x7nm represents a tetramer. The number of subunits (tetramers) per OAP can change between four and more than one hundred. AQP4 was described to occur as heterotetramers (Nicchia et al., 2010) reflecting the relative expression level of the different splice variants (M1 and M23; Neely et al., 1999, see below). The distribution of the inward rectifier potassium channel Kir 4.1 and the K<sup>+</sup> conductivity is similar to that of AQP4 and the dystrophin-dystroglycan complex (DDC) (Blake & Kröger, 2000; Amiry-Moghaddam & Ottersen, 2003; Connors et al., 2004; Nagelhus et al., 2004; Warth et al., 2005; MacAulay & Zeuthen, 2010). These molecules participate in the spatial buffering process of the extracellular space: synaptic activity and neuronal conductance evoke increase of the concentration of extracellular K<sup>+</sup> ions which are taken up by astrocytes. This K<sup>+</sup> uptake is followed osmotically by water entry through water channels of the type AQP4 (which are not absent in synaptic regions, but reduced in comparison with endfeet). In order to avoid swelling of astrocytes the water must be released into large extracellular spaces, and these are available around vessels and at the surface of the brain. It is exactly at this location, where AQP4 and also the inward rectifier potassium channel Kir4.1 are present for the directed extrusion of water and K<sup>+</sup> ions.

If this directed water flow is so essential for brain physiology, it may be expected to be closely regulated. Indeed, novel insights speak in favor of the hypothesis that the extracellular matrix heparansulfate proteoglycans have an important role for the insertion of AQP4 into the correct membrane domain. At present, at least two components of the BBB-ECM have been identified to be expressed during maturation of the BBB and which are not expressed by peripheral vessels, suggesting a specific role for the induction of the BBB: agrin (Barber and Lieth, 1997) and laminin (Hunter et al., 1992). The HSPG agrin was initially found to cluster acetylcholine receptors at the motor endplate (Nitkin et al., 1987; Bezakova and Ruegg, 2003) and described to participate also in maintaining the integrity of the BBB (Barber and Lieth, 1997; Berzin et al., 2000). Agrin is present in the subendothelial basal lamina (Barber and Lieth, 1997) and has a binding site to  $\alpha$ -dystroglycan (Gee et al., 1994), like laminin. Laminin is deposited in the basal lamina of CNS vessels, upregulated at the onset of BBB-maturation, and it is also localised in the neuromuscular endplate (Sanes et al., 1990). Furthermore, it is expressed by astrocytes and secreted as a soluble factor into the medium by cultured cells (Chiu et al., 1991). In addition, laminin participates in the correct positioning of the K<sup>+</sup> channel Kir4.1 and AQP4 in the astrocyte endfoot membrane (Guadagno and Moukhles, 2004).

### 3. The BBB in gliomas

#### 3.1 General alterations of the BBB in gliomas

Brain tumours, in particular the most malignant human glioblastoma, are characterized by pronounced hypercellularity, pleomorphism, numerous mitoses, foci of central necrosis, and excessive vascularization (Fig. 3a,b, 4a,b).

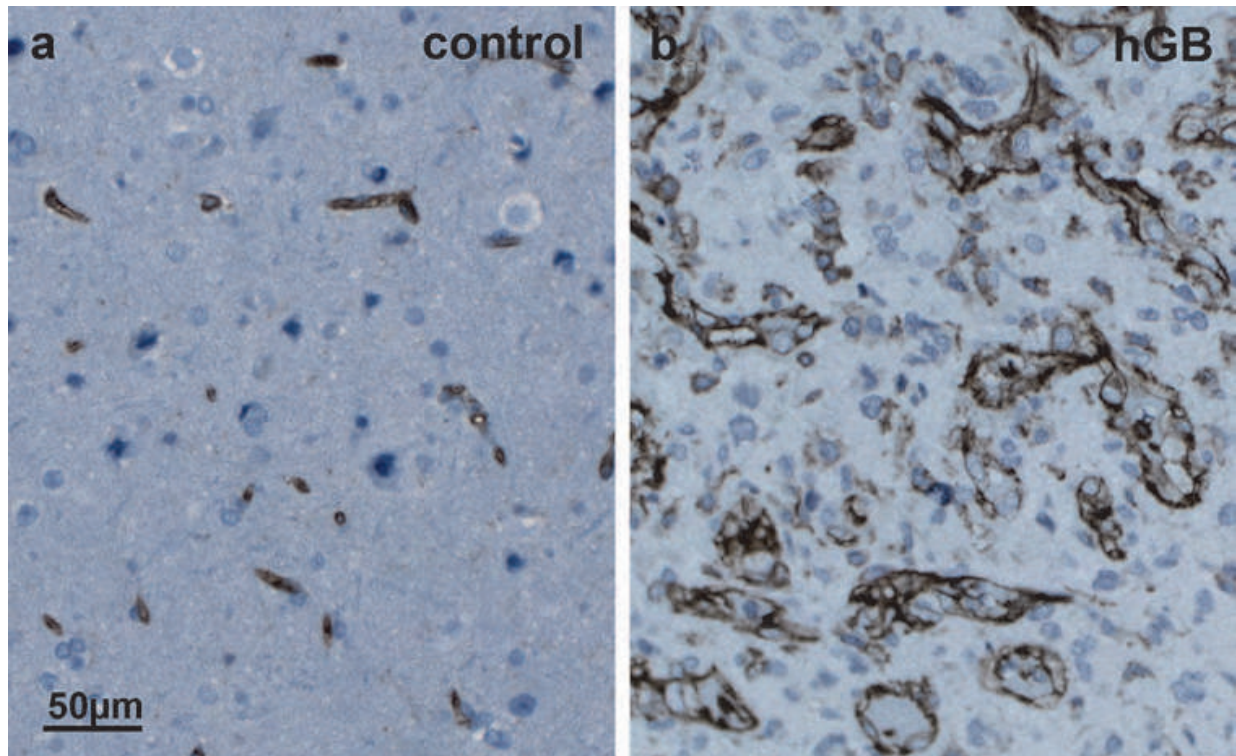


Fig. 3. Immunohistochemical staining of control (a) and glioblastoma tissue (b) of human brain (hGB) with an antibody against PECAM. Note the extreme difference of blood vessel structure in both tissues.

The literature on morphological alterations of the tumour blood vessels is extremely extensive (Hirano and Matsui, 1975; Dinda et al., 1993; Bertossi et al., 1997) but mainly related to the formation of fenestrations, the alterations in the number of caveolae and mitochondria, the thickness of the subendothelial basal lamina, the increase of the perivascular space, and to the pericytes. Previously, barrier-related molecular alterations in the capillary endothelial cells vascularising glioblastoma have been described (Liebner et al., 2000). The lost barrier function could be seen in magnetic resonance imaging (MRI) by contrast medium (CM) application (Sage & Wilson, 1994). The standard CM gadolinium is not able to cross the intact BBB, but the compromised barrier in glioblastoma. Low grade astrocytomas (WHO Grade II and III) are less aggressive than glioblastomas (WHO grade IV). Vessels of astrocytomas appear mostly normal and show rarely dysfunction of the BBB, which can be seen at MRI images. WHO grade II astrocytomas show no or little CM enhancement. WHO III grade astrocytomas enrich more CM than WHO grade II, but mostly less than glioblastomas. WHO III grade astrocytomas enrich more CM than WHO grade II tumours but much less than glioblastomas (Larsson et al. 1990). Pronin et al. (1997) found that edema production is quantitatively related to the degree of breakdown of the BBB as determined by gadolinium enhancement. The results of this group implied that the origin of the edema is in the area of the impaired BBB. Some drugs for example hypericin which normally can't cross the intact BBB are able to reach the main bulk of gliomas in rats through the disturbed BBB (Noell et al. 2011). The difficulty of treatment is how to reach single tumour cells in the infiltration zone where the BBB is not or less altered. It is easier to get drugs into the main centre of a high grade tumour than into the brain with an intact or less altered BBB.



### 3.2 Tight junctions in glioblastoma

As we have seen, the healthy BBB is dependent on a complex composition of tight junction molecules which obviously must be steadily maintained by the microenvironment, first of all by the highly polarized astrocytes. The astrocyte polarization in turn is evoked by local clustering of water and  $K^+$  channels in endfoot membranes by means of ECM compounds such as agrin. There is a mutual interrelationship between glial polarity and endothelial barrier, but it is not clear whether the endothelial TJs evoke glial polarity or *vice versa*. In an *in vitro* study, Tao-Cheng et al. (1990) were able to co-culture astrocytes and endothelial cells and to observe an accumulation of OAPs in the astrocyte membranes where they contacted endothelial cells. In human glioblastoma, Wolburg et al. (2003) described a loss of claudin-3 from the BBB (Fig. 5). In addition, we observed a thinning of the tight junction network which, in addition, was associated with the E-face (Liebner et al., 2000). Up to date, there is insufficient knowledge about the link between the detachment of astrocytes from the vessels and the vessel basal lamina, and down-regulation of tight junction components. Rascher et al. (2002) reported on a loss of the anti-agrin immunoreactivity in glioma vessels. Furthermore, a strict correlation of the expression patterns of occludin and agrin was described: Vessels without agrin-immunoreactivity revealed a loss of occludin from the tight junctions. This supported the suggestion that occludin as an important regulatory tight junction component is dependent on the presence of agrin.

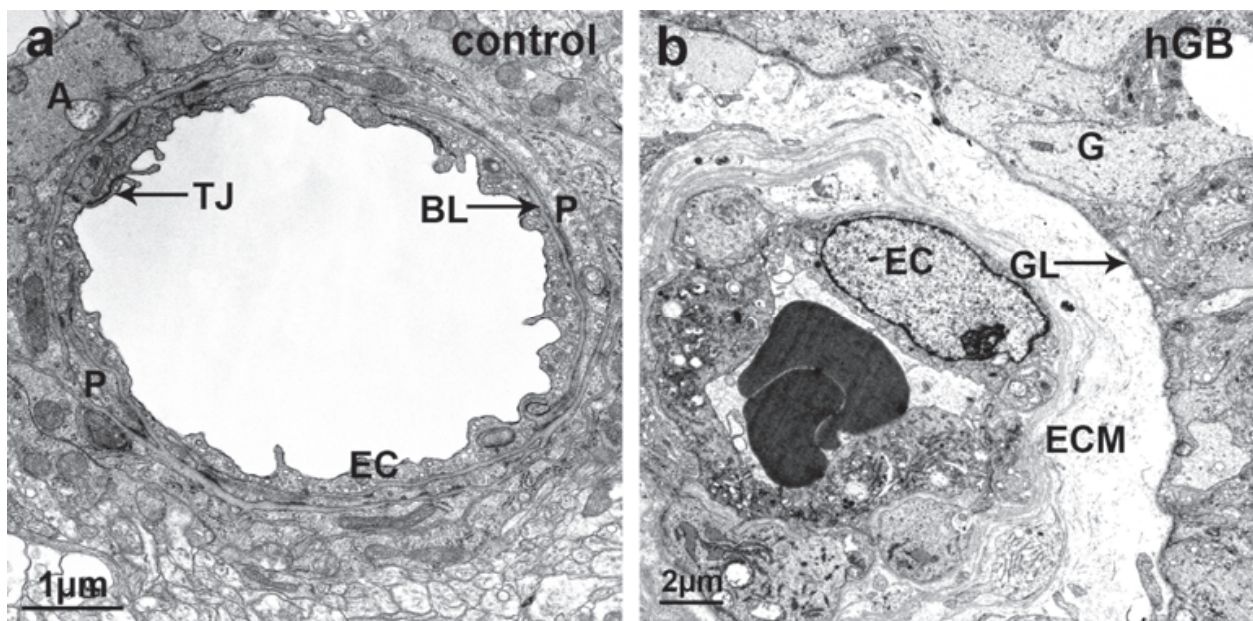


Fig. 4. Conventional electron microscopy of healthy brain (a) and human glioblastoma (b; hGB). Whereas normal blood vessels are intimately integrated into the neuropil, in brain tumour they are separated by a large extracellular space filled with extracellular matrix (ECM) substances. A astrocyte, BL basal lamina, EC endothelial cell, G glioma cell, GL glial limiting membrane, P pericyte, TJ tight junction

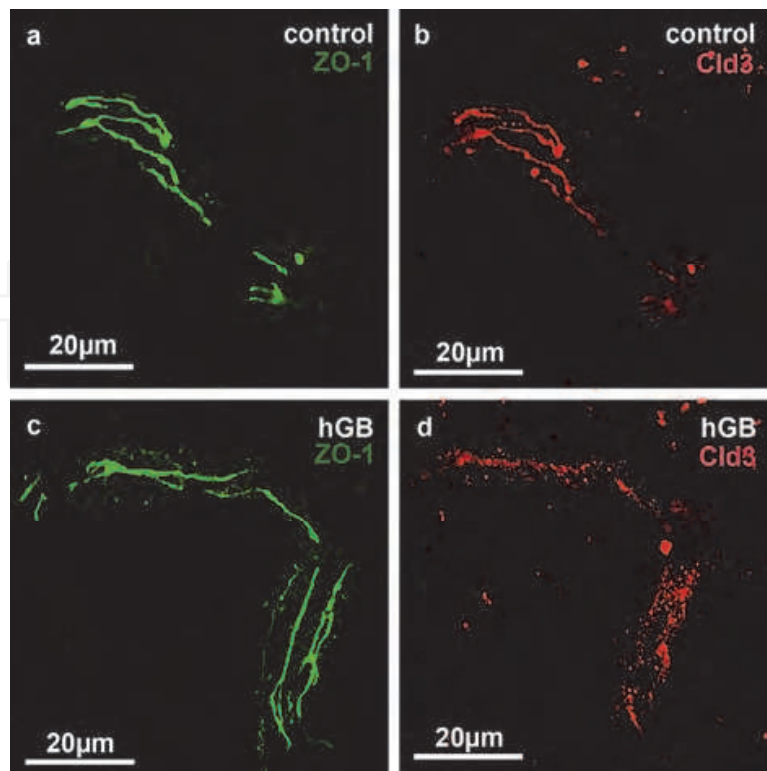


Fig. 5. Immunohistochemical stainings of tight junctional proteins ZO-1 (green) and claudin-3 (Cld3; red) in normal brain tissue (a,b) and in human glioblastoma (hGB; c,d). Note that the staining of claudin 3, but not of ZO-1, is disturbed, at places even missing, in glioblastoma.

### 3.3 Aquaporin 4 in glioblastoma

In human brain tumours, we see an increase of the perivascular ECM (Liebner et al., 2000). Absence of agrin (in the agrin-knockout mouse) has been described to evoke redistribution of AQP4 over the cellular surface (Noell et al., 2009), and loss of agrin (in the tumour) has the same consequence: AQP4 was no more restricted to vessel-directed membrane domains, but visible in all other membrane domains as well (Noell et al., submitted; Figs. 2, 6). Correspondingly, OAPs could be detected not only in membranes directed to blood vessels, but also in parenchymal membranes. In addition, AQP4 was described several times to be up-regulated in brain tumours (Saadoun et al., 2002; Warth et al., 2004). There is a remarkable inconsistency in the occurrence of AQP4 immunoreactivity and OAPs: In healthy brain tissue, only the OAP-crowded endfoot membrane is immunoreactive against AQP4. The parenchymal membrane is immunonegative. In the tumour, the whole glioma cell is strongly AQP4-immunopositive but the density of OAPs, even near vessels, is far below the density in the normal endfoot membrane. Therefore, there is only one conclusion: in glioma cells, AQP4 must also occur as a non-OAP molecule. We are far from understanding the functional difference between AQP4 in the form of arrays and AQP4 not in the form of arrays. Furman et al. (2003) have shown the freeze-fractured membranes of cells transfected with the AQP4 isoform M23, M1 and a mixture of M1 and M23. At the N-terminus of the protein, M1 is 22 amino acids longer than M23 (Jung et al., 1994). The M23 transfected cells formed huge lattices, whereas M1 transfected cells formed no arrays. Only the transfection of both isoforms resulted in the formation of OAPs resembling those in

astrocytes. However, the expectation that in glioma the M1 isoform would be specifically upregulated to explain up-regulation of AQP4 along with the down-regulation of OAPs was not verified: western blotting did not show any alteration of the ratio between both isoforms (Noell et al., submitted). A relationship between AQP4 expression and migration of astrocytomas cells has been postulated (Auguste et al., 2007). AQP4 was described to facilitate the infiltration of malignant cells in glioblastoma. This may suggest that an inhibition of AQP4 would limit this infiltration rate. However, there is no validated study to prove the specific pharmacological inhibition of AQP4. As well, there is neither any information on the influence of putative inhibitors on the AQP4 isoforms M1 and M23, nor on the link between water transport inhibition and the inhibition of migration of tumour cells (Zelenina, 2010).

### 3.4 Agrin in glioblastoma and its regulation by matrix metalloproteinases

In this section, we focus on the loss of agrin in brain tumour more closely (Fig. 6). One possible explanation is the gene-controlled down-regulation of agrin, however, there is no evidence for this assumption. More likely, agrin loss is a result of its degradation by matrix metalloproteinase 3 (MMP3; see below).

The MMPs are a growing family of degrading enzymes, which are associated with tumour cell invasion and blood vessel transmigration (for review see Nelson et al., 2000). MMP-2 (gelatinase A, type IV collagenase, 72kDa gelatinase), MMP-9 (gelatinase B, type V collagenase, 92 kDa gelatinase) and MMP-12 (metalloelastase, macrophage elastase) have been found to be upregulated by glioma cells, and MMP-9 and MMP-2 are secreted by proliferating glioma endothelial cells (Raithatha et al., 2000; Forsyth et al., 1999, Kachra et al., 1999). Basic fibroblast growth factor and vascular endothelial growth factor induce the release of MMP-9 in glioma cells *in vitro* in a dose- and cell density-dependent manner, implicating possible effects on these growth factors to enhance MMP-9 expression levels in gliomas (Tamaki et al., 1998).

Proteases may be involved in BBB-impairment in three different ways. 1. Shedding of growth factors which have been stored in the vessel ECM contributing to angiogenic processes. 2. Remodeling of the vessel ECM via stimulation of integrin receptors by binding to RGD binding sites.  $\alpha_v\beta_3$  integrin was demonstrated to be upregulated in glioma endothelial cells (Gladson, 1996), and its binding to these domains is able to induce cell spreading, migration, and angiogenesis. 3. The cleavage of the basal lamina which destroys the functionality of certain ECM-components (including agrin) which are thought to be important for the BBB integrity (Candelario-Jalil et al., 2009). Interestingly, we have found that laminin, in contrast to agrin, was not degraded in different gliomas (Fig. 6a,b).

Although it has been well-known for a long time that MMPs cleave all compounds of the extracellular matrix including agrin, reports by VanSaun and Werle (2000) in the muscular system and of Solé et al. (2004) in the CNS are still the only studies that have focused on the cleavage of agrin by MMP-3 (also called stromelysin-1). In the context of this review concerned with the role of agrin in BBB and absence of agrin in BBB deterioration in human brain tumours, the presence or regulation of MMP-3 within the perivascular complex seems to be of eminent importance (Fig. 7). Indeed, in cerebral ischemia (Candelario-Jalil et al., 2009), and in multiple sclerosis patients (Rosenberg et al., 1996; Kanesaka et al., 2006), MMP-3 was found to be upregulated and its level increased in the serum. In addition, an inhibitor of MMPs prevented MMP-induced tight junction degradation (Yang et al., 2007). Finally, in the MMP-3 knockout mouse, the lipopolysaccharide-induced opening of the BBB was less



pronounced and the tight junction proteins claudin-5 and occludin were less degraded than in the wild-type mouse (Gurney et al., 2006) suggesting that not only agrin is a substrate of MMP3, but tight junction molecules as well.

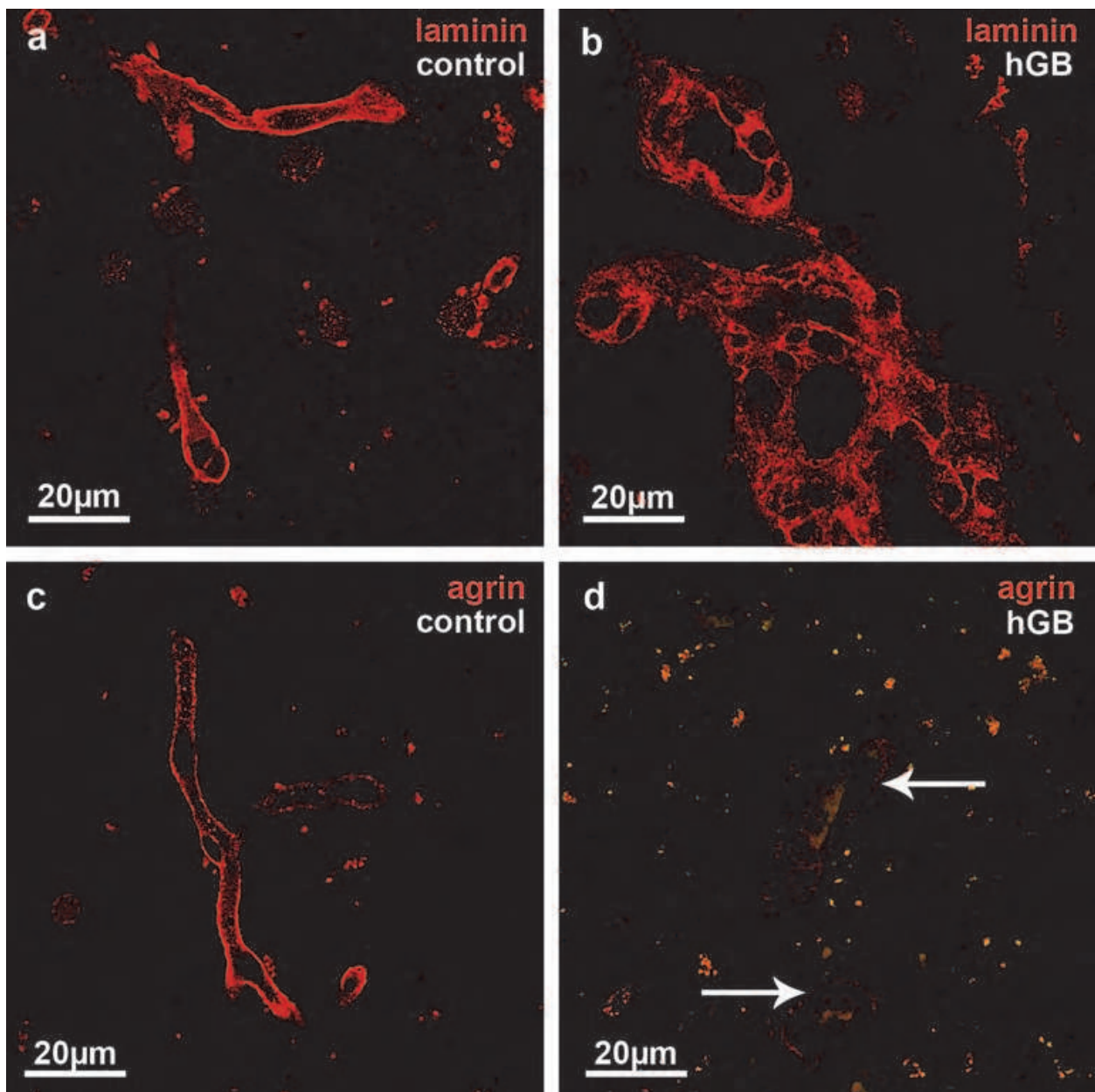


Fig. 6. Immunohistochemical staining of blood vessels in normal (a,c) and human glioblastoma (hGB; b,d) using antibodies against laminin (a,b) and agrin (c,d). Whereas laminin was not degraded in hGB in relation to control (a,b), agrin is heavily expressed around blood vessels under normal conditions (c), but the immunoreactivity has partially lost or reduced under glioma conditions (arrows in d).

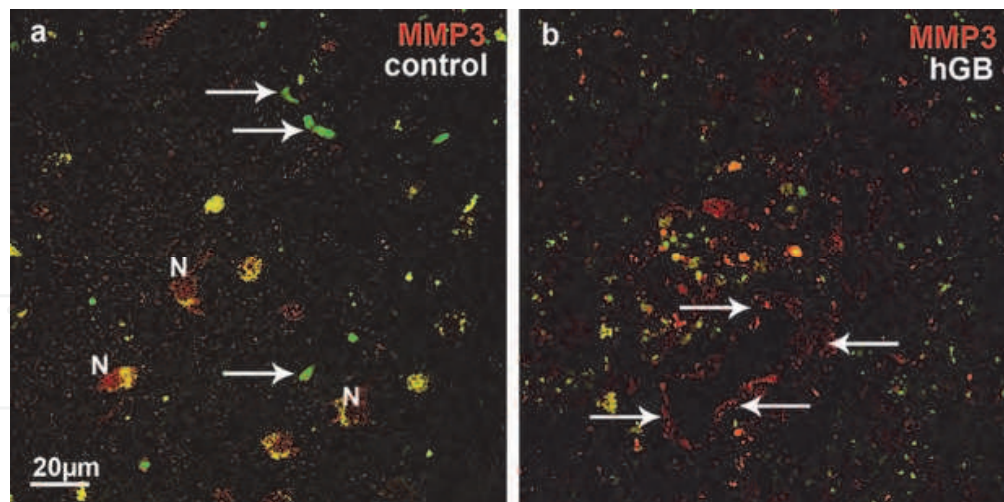


Fig. 7. Immunohistochemical staining of control (a) and glioblastoma tissue (b) of human brain (hGB) using an antibody against the matrix metalloproteinase 3 (MMP3, red). In normal tissue, MMP3 is expressed by neurons (N) and not by endothelial cells (white arrows in a, showing green autofluorescence), in glioblastoma MMP3 is upregulated by endothelial cells (arrows in b).

Interestingly, we have found in glioblastoma a mutual expression pattern of agrin and MMP3. Thus, the loss of agrin in the glioblastoma (Fig. 6c,d) may be explained as a degradation process dependent on the up-regulation of MMP3. Whereas MMP3 normally is expressed in neurons and, under conditions of ischemia/reperfusion, in oligodendrocytes, microvessels and microglia as well (Solé et al., 2003; Candelario-Jalil et al., 2009), we found in primary tumour a positive staining of MMP3 around blood vessels suggesting release of MMP3 into the perivascular space (Noell et al., submitted). Accordingly, where MMP3 was highly expressed the MMP3-substrate agrin could not be detected. The MMP3-inhibitor TIMP1 was detectable in NeuN-positive neurons, not in GFAP-positive glial cells. In normal tissue, there is an equilibrium between MMP3- and TIMP1-expression. This equilibrium which is carried by neurons is assumed to be disturbed in the glioblastoma, because neuronal loss leads to a decline of TIMP-1, but not of MMP3. It should be stressed that “the exact molecular mechanisms through which active MMP3 activates microglial cells remain to be clarified” (Candelario-Jalil et al., 2009). The disturbance of the MMP3/TIMP1-equilibrium in glioblastoma may be one of the factors leading to an increased degradation of agrin.

The concept that at least agrin is responsible for the correct targeting of AQP4-based OAPs to endfeet membranes has to be scrutinized for its validity in the *in vivo* situation. As described above, agrin has an effect on the assembly of AQP4 molecules in the membrane (Noell et al., 2007, 2009). Therefore, loss and degradation of agrin in glioblastoma must have disastrous consequences such as polarity loss and consecutive edema formation.

#### 4. Conclusions

The present overview is characterized by the detailed description of two systems bridged by a not well-defined and still shaky connection: the tight junction molecules of the brain microvessel endothelial cells representing the barrier proper, and the non-endothelial and extracellular molecules which are responsible for the regulation of the endothelial barrier.

Both systems are extremely complicated *sui generis* and increase their complexity by a crosstalk which, however, is poorly understood at present. Nobody knows the differential impact leading to barrier commitment, nothing is known about the links between extracellular matrix and barrier regulation, the pathway from integrins to the claudins or the precise role of astrocytes or astrocytoma cells in barrier formation or dysregulation, respectively. There is no doubt that the molecular analysis of the BBB is of outstanding clinical relevance, since insight into regulatory mechanisms of the paracellular barrier in the brain are of primary significance for the development of new therapeutic strategies. Treatment of brain tumours has to consider both tumour angiogenesis and the permeability of tumour vessels. The essential point of this contribution was the role of the extracellular matrix for the polarity of astrocytes, the loss of this polarity in glioma cells due to the increased activity of MMP3 followed by the degradation of agrin, and the resulting incapability of the glioma cell to directed release of water out of the interstitial space. Both are intimately dependent on the brain microenvironment, and in future research the analysis of this microenvironment will be the greatest challenge in understanding the blood-brain and blood-tumour barrier.

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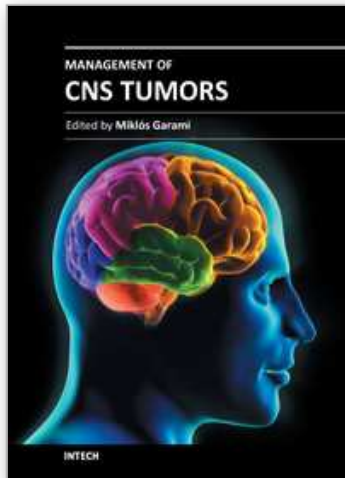
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