Chapter 4
The Blood-Brain Barrier in Glioblastoma: Pathology and Therapeutic Implications

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Abstract Glioblastoma (GBM) is a highly malignant form of brain tumour for which the prognosis is generally very poor and treatment options are limited. GBM is associated with rapid and aggressive tumour growth with associated cerebral oedema. Central to the difficulty associated with treating GBM is the challenge of getting chemotherapeutic drugs to cross the blood-brain barrier (BBB). Although vasculature within and around a GBM becomes more permeable due to pathological changes in the BBB, large areas of the tumour remain resistant to systemically administered agents. Here, we will introduce the concept of the BBB and its normal role in the healthy brain before describing how it becomes compromised in cases of GBM. This will cover physiological, genetic and functional aspects of BBB function and dysfunction. Finally, the therapeutic implications of modulating BBB permeability and receptor-mediated transport will be discussed with a focus on chemotherapeutic drug delivery.

Keywords Blood-brain barrier • Brain • Glioblastoma • Tumour

Abbreviations

BBB	Blood-brain barrier
GBM	Glioblastoma
NVU	Neurovascular unit
TJ	Tight junction

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4.1 Introduction

In order for the central nervous system to work effectively and efficiently, it requires a tightly regulated means of supplying neurons with nutrients and removing unwanted substrates from the cerebrospinal fluid (CSF). This is achieved by dense vascularisation of the cerebral parenchyma [1] where controlled trafficking of molecular species between the central nervous system and the periphery can occur. Access to the central nervous system is restricted by the blood-brain barrier (BBB), a complex interface between the blood stream and the cerebral parenchyma [2]. The presence of tight junctions and transporter proteins allows the BBB to selectively regulate the passage of molecules to and from the brain [3–5].

Glioblastoma (GBM) is a particularly malignant form of brain tumour for which the prognosis is generally poor and treatment options are limited [6]. GBM is associated with rapid and aggressive tumour growth with an associated cerebral oedema, and prognosis is poor for patients diagnosed with the condition [7]. Part of the difficulty in treating in GBM is the challenge of getting chemotherapeutic drugs to cross the BBB, even though the BBB in GBM becomes more permeable due to pathological changes in the BBB [8–11]. However, dynamic changes in BBB permeability can be achieved by targeting these tight junctions and transporters with suitable treatments [12]. This can be used to increase the efficacy of drug delivery [13] or removal of pathological material from the cerebral parenchyma, for example, the removal of amyloid beta in Alzheimer's disease [14].

The aim of this chapter will be to introduce readers to the BBB and its normal role in the healthy brain before describing how it becomes compromised in cases of GBM. This will cover physiological, genetic and functional aspects of BBB function and dysfunction. Finally, the therapeutic implications of modulating BBB permeability and receptor-mediated transport will be discussed with a focus on chemotherapeutic drug delivery.

4.2 The BBB in Normal and Pathological Conditions

Normal brain function requires rapid and controlled access to metabolic resources and molecular products from the periphery. At the same time, removal of unwanted material from the brain to the periphery is also vital. As such, the brain has evolved a pervasive and efficient vascular system that permeates through it; it is estimated that a neuron is never further than 20 μm from a capillary. Recent 3D imaging of the mouse brain shows that penetrating vessels with a diameter of approximately 23 μm access the tissue before branching into microcapillaries as small as 3 μm in diameter, ensuring a dense vascularisation of the brain [1]. The issue of supply of necessary metabolites and removal of waste is well catered for by this extensive microcapillary system, and access to the cerebral parenchyma is restricted by the BBB so that dangerous biochemical species cannot cause damage to delicate neuronal tissue.

The BBB itself is composed of a layer of endothelial cells lining the lumen of the capillary in order to create an interface between peripheral circulation and the cerebral parenchyma where substances can then enter the cerebrospinal fluid (CSF). Endothelial cells control transcellular transport from the blood into the CSF, expressing transporters for molecules that are essential for normal metabolism and homoeostasis such as glucose, insulin and amino acids [3, 5]. The endothelial cells form adherens junctions and tight junctions between each other in order to limit paracellular transport between the vascular and cerebral compartments [12]. Interendothelial adherens junctions are mainly composed of members of the cadherin family such as vascular endothelial (VE)-cadherin and N-cadherin [15]. Tight junctions are composed of about 30 proteins including occludin, tricellulin and members of the claudins and junction adhesion molecule families [16, 17]. Tight junction proteins are anchored to the intracellular cytoskeleton of the endothelial cells by transmembrane proteins such as zonula occludens-1 (ZO-1) [18]. Tight junctions restrict molecules with a size greater than 400–450 Da from passing between endothelial cells [19, 20]. Furthermore, movement of small ions across the BBB is also restricted given its electrical resistance, on average around 1870 omega (Ω) cm^2 [21]. Taken together, the endothelial cell layer can regulate access to and from the central nervous system to a high degree, with the result that approximately 98 % of drugs developed to target neurological disorders being unable to cross the BBB [22].

The microcapillaries that supply the brain are surrounded by a group of different cell types that form the neurovascular unit (NVU), including neurons, astrocytes, microglia and pericytes. The non-neuronal cells of the NVU act both as scaffolding and as mediators of molecular transport into and out of the brain. Within the NVU, contractile pericytes form layers on the abluminal surface of the microcapillaries, regulating permeability in the mature BBB [23]. Pericytes are covered by a macromolecular layer known as the basal lamina which is in turn enclosed by perivascular endfeet from astrocytes to create a supportive sheath (the glia limitans) around the microcapillaries that supply the brain [24–26]. Microglia then form a line of protection on the brain side of the BBB, able to mount an immune response should an unwanted substance make it through the BBB [27].

Although the cerebral endothelial cell layer is the main workhorse of the BBB, the pericytic and glial support structure surrounding the microvasculature is necessary to the development and maintenance of BBB integrity and functionality. Development of the BBB is achieved by radial glia first via the production of retinoic acid to induce BBB formation and secondly by stabilisation of developing vasculature through Wnt signalling [28]. At the same time, interactions between pericytes and endothelial cells functionally regulate BBB integrity [29] via signalling involving transforming growth factor β (TGFβ) [30], platelet-derived growth factor B [31] and the forkhead transcription factor Foxf2 [32]. Once the BBB is mature, astrocytes form endfoot projections that can modulate permeability—possibly through TGFβ [33] and angiotensin signalling [34]. In the NVU, astrocytes act as a go-between for neurons and vasculature, synchronising cerebral blood flow, maintaining homoeostasis and regulating water content in the cerebral parenchyma [25]. Astrocytic endfeet are known to regulate transport of Na^+ and Cl^- across the endothelial cell layer [35] through intercellular Ca^{2+}

signalling between astrocytes and endothelial cells [36]. Astrocytic endfeet also regulate neurovascular coupling via a nitric oxide-dependent intracellular Ca^{2+} signalling cascade [37], ensuring that metabolic supply is maintained during neuronal activation. Pericytes also play a role in controlling the environment within the NVU through the regulation of cerebral blood flow [38] and by coordinating astrocytic endfoot position on the walls of the cerebral vasculature [39].

It is important to note that the BBB is not homogenous; different regions show variations in permeability. For example, the circumventricular organs entirely lack a layer of endothelial cells and have almost free access to peripheral blood flow [40, 41]. Even where an endothelial cell layer is well established, the permeability of the BBB is not a static value as BBB permeability is a dynamic process with up- and down-regulation of tight junction proteins occurring constantly [12]. Diurnal and seasonal effects on BBB permeability have been described [42, 43], and recent work from our group shows that concentrations of molecular regulators of BBB permeability follow a circadian rhythm (unpublished data). This reinforces the idea that the BBB is a constantly changing entity under normal conditions and this dynamism requires careful consideration in addressing questions about pathological processes and therapeutic interventions.

4.3 The Compromised BBB in GBM

GBM is a malignant class of Grade IV tumours that tends to form in the brain or spinal cord, mainly in adults aged 50–60 [44]. The prognosis for GBM is poor in many cases given the aggressive growth of the tumour and difficulties in treating GBM with standard oncological treatments [45, 46]. This means that the five-year survival of patients diagnosed with GBM is only 1.9 % in patients undergoing radiotherapy alone [7]. Typically, abnormal differentiation of brain tissue results in a mass of cancerous tissue though the exact source of the initial insult is under debate. It has been suggested that GBMs develop from aberrant glial cells but more recently the idea that cancer stem cells (CSCs) are responsible for the condition. These CSCs develop from a suitable progenitor cell line where the normal developmental cascade has been altered leading to unregulated growth [44]. Given the fact that GBMs tend to form numerous cell types during their growth, it is likely that multipotent progenitor cells are at fault in this condition [47–50] and the location of GBM development within the brain may further influence the fate of these CSCs [51, 52]. In particular, co-activation of the Ras and Akt pathways has been identified as being necessary for GBM induction [53, 54]. The role of Ras is confirmed by the presence of altered Notch signalling in GBM cell lines [55]. Ras has also been implicated in the maintenance of GBM with suppression of Kras expression resulting in GBM apoptosis and regression in a mouse model of GBM [56].

Tumour growth and maintenance are facilitated by newly formed blood vessels that give the cancerous cells access to the peripheral blood supply; a well-established hallmark of higher-grade brain tumours is an extensive network of microcapillaries

within the cancerous region [57–59]. Out of all brain cancers, GBM shows a relatively high level of biomarkers relating to proliferation and angiogenesis [60], which is unsurprising given the aggressive nature of GBM. There seems to be a reciprocal relationship between the developing tumour and the vasculature of the brain; there are high levels of perivascular nestin-positive CSCs in the early stages of GBM growth [61], nestin being a marker of angiogenesis that is upregulated in cancerous cells [62]. Calabrese and colleagues (2007) also describe direct interaction between cultured CSCs and endothelial cells and, most importantly, that endothelial cells promote GBM development in vivo. This is a subversion of the regulatory role of endothelial cells on normal neural stem cell development [63].

A number of molecular pathways regulating angiogenesis in GBMs have now been identified. Vascular endothelial growth factor (VEGF) in particular stands out as an important regulator of angiogenesis [64]. CSCs actively promote VEGF signalling, directly acting on local endothelial cells to promote angiogenesis [65]. Normally, endothelial cells do not express the tyrosine kinase receptor for VEGF, but it is expressed on endothelial cells associated with tumour formation [64]. Inhibition of VEGF signalling following transfection of human GBM cells into the brains of nude mice was subsequently shown to inhibit GBM growth and decrease the rate of angiogenesis in vivo [66]. Specific targeting of the VEGF tyrosine kinase receptor using a diphtheria toxin conjugated to tumour-specific isoforms of the VEGF receptor has been also shown to prevent tumour-associated angiogenesis and inhibit GBM growth in vivo [67–69]. Translation of anti-VEGF treatments to human clinical cases of glioma has shown therapeutic promise; however GBM still remains resistant to treatment even with these new therapies [70–72]. Part of this resistance may be due to the issue with invasive cells that migrate away from the GBM core where vascularisation is at its most dense [73], a process that is itself dependent on VEGF signalling [74].

These new blood vessels develop a BBB but one that shows marked differences compared to that in normal tissue [4, 75] with alterations of both adherens [10, 11] and tight junction proteins [8, 9]. Additionally, epigenetic modulation of GBM development [76] and GBM's susceptibility to radiation therapy [77] are associated with changes in the expression of markers for tight and adherens junctions, indicating an intimate relationship between endothelial integrity and GBM growth. It is well established that switching of cadherin expression in the adherens junction is involved in GBM development [78, 79] and these alterations have knock-on effects on tight junction stability [80, 81].

At the level of the tight junction, there is almost complete loss of claudin-1 [9] and reduced levels of claudin-3 [82], claudin-5 and occludin [9] associated with GBM. At the same time, decreased levels of claudin-1 and claudin-5 in human GBM samples are accompanied by significant increases in the expression of the adherens junction protein β-catenin [83]. Liebner and colleagues (2000) also describe alterations in plakoglobin and beta-catenin, further suggesting abnormally formed tight junctions in this pathological state. In clinical cases of GBM, these regions of abnormal tight junctions can be identified through contrast magnetic resonance imaging (MRI); a contrast agent (gadolinium) is injected into the patient

and areas of high BBB permeability show a hyperintensive signal in T1-weighted scans [84, 85]. Using this method, regions of increased BBB permeability have been identified in patients [86], fitting with the molecular data from preclinical studies outlined above. This loss of tight junction integrity can be reversed in vitro using an anti-TGFβ antibody [4]. Interestingly, there appears to be a connection between TGFβ effect on endothelial cells and angiogenesis; in vitro analysis shows that TGFβ upregulates VEGF and inhibition of TGFβ signalling leads to an increase in claudin-5 levels [87], and human neuroimaging suggests that the extent of BBB "leakiness" is an indicator of patient survival [85].

Abnormal tight junctions in GBMs are associated with changes in basal lamina composition, namely, decreases in agrin, a basal lamina protein associated with BBB function, and increases in tenascin, which is normally absent in the basal lamina [88]. Data from an in vitro model of BBB using cultures of rat endothelial cells suggests that there may be a transient decrease in BBB permeability given that there is a fibroblast growth factor-2-dependent increase in occludin and ZO-1 protein levels following initial exposure to human GBM cells accompanied by an increase in transendothelial electrical resistance (TEER) [89]. However, this may be a transient increase in tight junction efficiency that is lost as the GBM becomes established in the tissue.

The role of the BBB in GBM is of further clinical significance when considering how disruption in fluid clearance can lead to serious cerebral oedema [90]. Under normal conditions, the BBB is responsible for regulating osmotic processes via the aquaporin family of proteins [91, 92]. In particular, aquaporin-4 has a clear role in water transport; it is the most abundant water channel in the central nervous system and is found throughout the glia limitans in the astrocytic endfeet lining the BBB [93]. Aquaporin-4 is implicated in multiple regulatory processes including, but not limited to, regulation of extracellular space volume, circulation of CSF, waste clearance and cell migration [94]. Importantly, directly disrupting aquaporin-4 function using aquaporin-4-immunoglobulin G causes a significant increase in BBB breakdown [95].

As noted above, the BBB becomes disturbed within the GBM and cerebral oedema has been identified in regions neighbouring the GBM [86]. Changes in aquaporin expression have been described during brain tumour development, namely, increases in aquaporin-1 [96] and aquaporin-4 [97]. Aquaporin-4 has been shown to be responsible both for the induction of cerebral oedema and for its resolution in a number of pathological states [98, 99]. Nevertheless, even though aquaporin-4 expression increases along with levels of cerebral oedema [100], aquaporin-4 levels are not predictive of patient survival and may follow other processes involved in tumour growth rather than oedema itself [101]. This is supported by the association between increased aquaporin-4 expression in tumours and simultaneous increases in VEGF and hypoxia-inducible factor-1α [102], suggesting a link with angiogenesis during tumour development.

Increases in BBB permeability during GBM may also be linked to aquaporin expression; in GBM the expression of aquaporin-4 moves from its polarised configuration in the astrocytic endfeet [91] and instead covers the cell bodies of the cancerous cells leading to dysregulation of the BBB [103]. Complicating the matter,

aquaporin-4 seems to play a direct role in oedema during therapy against GBM; treatment with radiotherapy and chemotherapy modulates perivascular levels of aquaporin-4 leading to a reduction in cerebral oedema associated with GBM [104]. However, until recently there has been little work performed on targeting the aquaporin system directly in combating cerebral oedema in neuropathological disorders [99]. Other symptoms of GBM have been addressed by targeting aquaporins; successful attempts at modulating the aquaporin system have been made in order to attenuate angiogenesis, cell migration and growth in aquaporin-1-null mice [105] and following knockdown of aquaporin-4 expression in human cell cultures and in nude mice in vivo [106].

4.4 Therapeutic Implications of the BBB in Treating GBM

It must be considered that even though the BBB within the GBM is compromised, it is still largely functional and will continue to perform its job in excluding large molecules from the cerebral parenchyma [20]. This creates the challenge of targeting the GBM effectively using chemotherapies as many commonly used chemotherapeutic drugs are in the size range of 450–850 Da so the BBB will still greatly limit access of many therapeutic compounds to the brain [20, 22]. The current standard for GBM treatment is surgical resection [107] followed by radiotherapy with an adjuvant chemotherapy [48]. Nonetheless, response to chemotherapy in GBM is poor, meaning that therapeutic success is still very low [7], so any attempts at increasing the efficiency of drug delivery to the brain are desirable. Therefore, techniques to increase BBB permeability using osmotic, genetic and physical interventions or via receptor-mediated transport have been developed in order to aid in delivering drugs that would be too large to cross the BBB under normal circumstances.

Osmotic modulation of BBB permeability can be accomplished using a variety of techniques. The most commonly used approach is the use of a concentrated solution of the sugar mannitol to increase BBB permeability across the entire brain for several minutes [108, 109] with Na^{+}/Ca^{2+} exchange governing the length of time that BBB permeability is increased [110, 111]. 20 % mannitol was shown early on as a way to improve chemotherapy survival times [112]. However, its use in treating brain cancers has been controversial as osmotic modulation tends to cause widespread opening of the BBB with debatable effects on the therapeutic index of co-administered chemotherapeutic agents [113] and mannitol can induce seizures in patients with epileptiform activity persisting for days [114]. Therefore, mannitol has fallen out of favour as methods for bypassing the BBB with fewer side effects have been introduced. These newer approaches favour selective opening of the BBB; for example, localised BBB opening has been achieved using a convection-enhanced delivery of ethylamine-human serum albumin (EA-HSA) in order to allow greater access of systemically administered methotrexate to the cerebral parenchyma, resulting in reduced tumour growth and increased survival in a rat model of glioma [115].

RNA interference is a method of knocking down gene expression by delivery of a tailored piece of RNA via a viral or non-viral vector that shows great promise across a range of neurological disorders [116]. It has been used to directly target GBM as many of these vectors are designed to pass through the BBB [117–120]. However, RNA interference can also be used to alter BBB permeability in order to allow other therapeutic compounds to access the cerebral parenchyma. Global or selective genetic modulation of the endothelial tight junction can be achieved systemically or locally using short hairpin (sh) or small interfering (si) RNAs. Modulation of BBB permeability using siRNAs has proven to be of therapeutic use in a mouse model of Alzheimer's disease, knocking down occludin and claudin-5 levels increases the permeability of the BBB enough to allow significant clearance of amyloid beta from the brain to the periphery [14]. For GBM, this type of approach could aid in delivering drugs as large as 1 kDa from the periphery across the BBB without inducing oedema [121, 122], allowing many standard chemotherapy drugs to enter the cerebral parenchyma. Furthermore, RNA interference can be utilised to enhance delivery of drugs that are already small enough to pass through the BBB [123], meaning that a smaller dose is needed to be systemically administered which may reduce side effects associated with a particular drug. In terms of GBM and other brain cancers, RNA interference has so far been successfully used preclinically to knock down aquaporin expression in vitro and in vivo. Knockdown of aquaporin-4 significantly reduces water mobility under normal conditions in vivo [124] and significantly reduces GBM migration and growth by disrupting pathways involved in cell invasion and adherence [106].

A third way of altering BBB permeability is the use of physical means such as focussed ultrasound which has recently been shown to be a promising non-invasive method to treat a number of cancers including prostate cancer [125] and liver carcinomas [126] as well as ablation of brain tumours in humans [127–130]. Ablation using this method is problematic at present due to side effects with overheating of nontarget brain tissue. However, at lower intensities it can also be used as a way to increase BBB permeability in vivo [131] though this has yet to be attempted in humans [132]. A 1 MHz sonication pulse can significantly increase BBB permeability in tumours in rats [133–136], and using MRI to guide focussed ultrasound application, it is possible to selectively increase BBB permeability in precise target regions of the rat brain in vivo [137]. This can then be used to allow greater access to the brain for a number of therapeutic compounds including drugs and genetic therapies: uptake of doxorubicin [138] and temozolomide [139] is significantly increased following focussed ultrasound exposure and oligonucleotides, and DNA plasmid delivery can be made even more effective when focussed ultrasound was combined with nanoparticle delivery [140, 141]. Enhanced localisation of focussed ultrasound combined with targeted drug delivery can be achieved using microbubbles preloaded with the desired drug [142, 143].

Finally, receptor-mediated transport is a method of crossing the BBB without altering its baseline level of permeability; instead drugs are bound to a ligand that can normally cross the BBB unimpeded [144]. A number of suitable endocytotic receptors have been identified including low-density lipoprotein receptor-related

protein-1 and protein-2 (LRP-1 and LRP-2), transferrin receptor, insulin receptor and insulin-like growth factor receptor [144]. LRP-1 is found on endothelial cells and is responsible for transcellular transportation for multiple ligands across the BBB [145], and as such, it has become a target for the development of drug carriers that co-opt LRP-1 to carry therapeutic compounds across the BBB into the cerebral parenchyma [146]. Delivery of the chemotherapeutic drug doxorubicin and paclitaxel to the brain via LRP-1 has been achieved by binding doxorubicin to p97 [147] and by binding paclitaxel to angiopep-2 [148]. This significantly increases the effectiveness of GBM uptake of Adriamycin and paclitaxel in mouse models of glioma. Researchers have also taken advantage of transferrin's ability to guide material across the BBB (discussed in greater detail below) with doxorubicin having been successfully delivered in this manner [149].

Nanoparticles have also been used for many years to aid the delivery of drugs across the BBB [150, 151] via receptor-mediated transport involving apolipoproteins B and E [152–154]. Doxorubicin has been successfully delivered to the rodent brain by binding it to polysorbate-coated nanoparticles [155, 156]. Not only does nanoparticle-bound doxorubicin show effectiveness in treating GBM in preclinical experiments [157], the data also suggests that binding doxorubicin to polysorbate-coated nanoparticles also reduces the drug's systemic toxicity [158]. Similarly, methotrexate can be delivered to the brain using the same type of polysorbate-coated nanoparticles [159]; there was a significant decrease in tumour size and a significant increase in the rates of apoptosis in a rat model of glioma using an alternative nanoparticle system (methotrexate was loaded into lipid core nanocapsules) [160].

Combining RNA interference and receptor-mediated transport may result in better therapies, and research involving the transferrin receptor has seen convergence of these techniques leading to increased efficacy in the treatment of GBM [161, 162]. It has been long known that transferrin receptor levels are greatly increased in GBM [163] and these levels are significantly increased following radiotherapy [164], making them an attractive option for delivering drugs to cancerous brain tissue. Early in vitro research using transferrin conjugated to toxins showed that targeting the transferrin receptor could be a way to selectively target GBMs in vivo [165, 166]. Furthermore, RNA interference and traditional receptor-mediated transport can be made more efficient by conjugating transferrin onto nanoparticles; for example, spherical nucleic acids can be conjugated onto gold [167], cationic solid lipid [168] and hyaluronan-grafted lipid-based nanoparticles [169], whereas transferrin can be conjugated onto poly(lactic-co-glycolic acid) [170], poly(ethylene glycol)-poly(l-lactic-co-glycolic acid) [171] and gold nanoparticles [172].

Using oligonucleotides against laminin-8 (a vascular basement membrane protein that is upregulated during GBM) conjugated to an antibody against the transferrin receptor, significant decreases in GBM microvasculature density and significant increases in survival were obtained in nude rats [173]. Polypropylenimine dendrimers can be used as a non-viral alternative to deliver DNA to target cells [174], and conjugating these with transferrin has been shown to be effective in delivering treatments directly to cancerous cells with little toxicity [175]. This has allowed direct delivery of siRNA to GBM cells without using a viral vector [176].

It has also been demonstrated that conjugating microRNA to transferrin and to a nanoparticle delivery system can result in higher levels of transport across the BBB than transferrin alone [177].

4.5 Conclusion

The BBB's tight control over access to and from the brain becomes disrupted in the presence of GBM as expression of tight junction proteins decreases, leading to an increase in BBB permeability. Increases in angiogenesis help to nurture the GBM, and alterations in aquaporin-4 levels contribute to cerebral oedema around the tumour. Despite the compromised nature of the BBB within the GBM, delivery of chemotherapeutic drugs remains problematic. BBB permeability can be further increased by osmotic modulation, RNA interference and focussed ultrasound treatment. Alternatively, receptor-mediated transport can be used to "piggyback" into the cerebral parenchyma using the transporters naturally expressed on endothelial cells. This in turn can be facilitated by the use of nanoparticle conjugates.

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