The role of claudin-5 in blood-brain barrier (BBB) and brain metastases (Review)

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Received April 26, 2013; Accepted November 19, 2013

DOI: 10.3892/mmr.2013.1875

Abstract. Metastatic brain tumours are frequently observed in patients with lung, breast and malignant melanoma and a severe complication of metastatic cancers. With improved primary cancer treatments, including surgery, radiation therapy and chemotherapy, patients are now living longer following initial treatment, compared with previous treatments. Brain metastasis (BM) remains a significant clinical issue. Since BM represents a major therapeutic challenge, it is vital that the mechanisms of interaction between tumour cells and the blood-brain barrier (BBB), as well as the method by which tumour cells establish metastatic tumours in the brain, are understood. A key step in BM is the interaction and penetration of the BBB by cancer cells. The BBB consists of endothelial cells, pericytes, astrocytes and a number of molecular structures between these cells. The BBB relies on the tight junctions (TJs) that are present between the endothelial cells of the brain capillaries to provide a closed environment for the brain. TJs comprise a number of proteins, including occludin, claudins and junctional adhesion molecules (JAMs). Among them, claudins are the key integral proteins that regulate BBB permeability. It has previously been shown that claudin-5, not only regulates paracellular ionic selectivity, but also plays a role in the regulation of tumour cell motility, suggesting that TJs and claudin-5 contribute to the control of BM. This study reviews the role of claudin-5 in the regulation of BBB permeability during the brain metastatic process.

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Key words: blood brain barrier, brain metastases, tight junctions, claudins, claudin-5, permeability

Contents

1. BM

BM are the most life-threatening complications of cancer, the exact prevalence of which is not clearly known. The incidence of BM in the US population is ~170,000 new cases/year, with a prevalence of 8.3/100,000 (1,2). Clinical studies have shown that ~8.5% of cancer patients present with BM, but a biopsy study shows a significantly higher BM incidence rate of 8.7-26% in a cohort of patients with carcinoma (3,4). The majority of studies have demonstrated that the incidence of BM is equal to or 2-10 times that of the primary intracranial tumours (1-6). Incidence has increased with the availability of improved neuroimaging techniques that aid in the early diagnosis and effective systemic treatment regimens, leading to a prolonged life, thus allowing cancer to disseminate to the brain.

The primary tumours most likely to metastasize to the brain are located in the lung (50%), breast (15-20%), skin (melanoma) (10-15%), colon-rectum (2-12%), kidney (1-8%) and thyroid gland (1-10%). The primary site is unknown in <10-15% of the patients. Soft tissue sarcoma, childhood Ewing sarcoma and childhood rhabdomyosarcoma are also significant sources of brain metastases (4,7). Among these tumours, melanoma metastasizes to the brain with one of the highest frequencies. However, the most common sources of BM in children are osteogenic sarcoma, soft tissue sarcoma and germ cell tumours, which is different from those of adults (8).

Metastatic tumours most commonly invade the cerebral hemispheres (80%), cerebellum (15%) and brainstem (<5%) (7,9,10). This distribution is hypothesized to reflect the correlation between the distribution of tumours and cerebral vasculature. The mean age of presentation for brain metastases

is ~55-65 years. The clinical manifestation of BM varies with the location of the metastatic tumours. The most common symptom is headache, occurring in 24-53% of patients. This is followed by limb weaknesses, altered neurocognitive function, seizures and ataxia (7). The majority (70%) of patients with BM present with multiple lesions and only 30% present with a solitary lesion (8).

Comprehensive treatment for BM includes whole-brain radiation therapy, stereotactic radiosurgery, radiosensitizers, chemotherapy and surgery (7-9,11-14). Targeted therapies are providing promising results (5,15). The median survival time from time of diagnosis is only 1 month for *de novo* patients (7). Patients receiving whole-brain radiation therapy have a median survival of 4-5 months, while patients treated with multidisciplinary management have a survival time of ≤12 months (16). Since brain metastases represent a significant therapeutic challenge, it is necessary to understand the mechanisms by which tumour cells interact with the BBB in order to determine targets of prevention and treatment of BM formation.

The formation of BM is a complex series of processes in which detached primary tumour cells are transported through blood vessels, adhere to vascular endothelial cells, transmigrate through the BBB into brain parenchyma where they lodge and proliferate in the new location to form a secondary tumour mass (3,5,9). A number of key molecules and proteins regulate this process. The only route for BM is through the blood vessels, due to the lack of lymphatic duct in the central nervous system (CNS). Regardless of their source, metastatic tumour cells are capable of transmigrating through the BBB to invade the brain.

2. BBB

The BBB is a barrier between blood circulation and brain parenchyma, providing anatomical and physiological protection for the CNS, supplying brain tissue with nutrients, filtering harmful compounds from the brain back to the bloodstream and shielding the brain from toxic substances in the blood. The BBB consists of four primary cellular elements: cerebral endothelial cells (ECs), astrocyte end-feet, microglial cells and pericytes (17).

ECs lining the brain capillaries are thin, flat cells interconnected by TJs. Ultrastructure studies (17-20) demonstrated that these ECs have the following characteristics: ECs forming TJs at their adjacent margins, produced by the interaction of a number of transmembrane proteins projecting into the paracellular space and effectively sealing it, thus confining penetration across brain endothelium to transcellular mechanisms; endothelial cytoplasm lacking fenestrations typically present in peripheral-tissue capillaries; fewer pinocytotic vesicles compared with peripheral endothelial cells and more mitochondria, suggesting important metabolic activity (17-22). Cerebral endothelial cells share common features with other endothelia, including the presence of factor VIII, high alkaline phosphatase and γ-glutamyl transpeptidase activity, uptake of acetylated low-density lipoprotein and epithelia, high transendothelial electrical resistance (TEER), a continuous line of tight junctions and low level of pinocytosis, the latter being indispensable for the barrier function (11).

Pericytes are located in the duplication of the basement membrane, in close contact with endothelial cells. Gap junctions have been described between the two cell types (23). Pericyte-endothelial cell interactions were observed as being significant in the following areas: angiogenesis, BBB formation and maintenance, vascular stability and angioarchitecture, regulation of capillary blood flow and clearance of toxic cellular byproducts necessary for proper CNS homeostasis and neuronal function (24). Defects or absence of pericytes may lead to a number of CNS diseases, including neurodegeneration and neurovascular diseases and injury (25). Astrocytic end-feet almost completely ensheath the capillary walls, thereby covering endothelial cells, as well as the intimately associated pericytes (26). The coverage is not complete, allowing a direct contact of nerve endings with the basal membrane (27,28). Astrocytes are associated with BBB permeability and ionic and water regulation.

Microglia are another type of cell in close contact with cerebral vessels. Microglia are primary immune effector cells in the brain and spinal cord and are important in neuroinflammatory processes (29). These cells provide immune surveillance and are mobilized in response to disparate diseases and injuries. They are also essential defenders against a number of neurodegenerative diseases (29,30). However, the exact role of microglia in the neurovascular unit remains poorly understood and occasionally controversial.

In the human brain, neurons are generally not in direct contact with cerebral endothelial cells. It is currently unclear as to whether there are signals from endothelial cells to neurons and vice versa, which may be significant for brain homeostasis or neuronal function. The presence of neurotransmitter receptors on endothelial cells was observed in laboratory rats, suggesting a communication between the two cells.

Aside from these cells, there is a specialized extracellular matrix, the basement membrane, covering endothelial cells from the outside astrocytes and pericytes. Its primary protein components include collagen, particularly type IV, fibronectin, laminin, tenascin and proteoglycans. The extracellular matrix serves as an anchor for endothelial cells and modulates TJ protein expression. In addition, it is involved in the alteration in BBB permeability during the pathological process of brain tumour and cerebral ischemia (31,32). All the aforementioned components form the BBB which acts extremely effectively to: i) maintain the ionic composition optimal for synaptic signaling function; ii) protect the brain from neurotransmitters in the rest of the body; iii) prevent macromolecules from entering the brain; iv) shield the CNS from neurotoxins circulating in the blood and v) ensure an adequate brain nutrition supply. Thus, the BBB provides a stable microenvironment that is critical for complex neural function and protects the CNS from chemical insult and damage (33). Transport across the brain endothelium is strictly limited through a 4-fold defense line: paracellular barrier, represented by interendothelial junctions; transcellular barrier, assured by the low level of endocytosis and transcytosis; enzymatic barrier, including acetylcholinesterase, alkaline phosphatase, γ-glutamyl transpeptidase, monoamine oxidases and drug-metabolizing enzymes and the efflux transporters, ABC-B1, -C1, -C4, -C5 and -G2 (34). Small gaseous molecules, including O_2 and CO_2, freely diffuse through the lipid membranes which serves as a route of entry for small lipophilic agents, including barbiturates, nicotine and ethanol. However, specific blood-to-brain influx transport

systems exist to supply nutrients, including glucose, amino acids and nucleotides, which cannot freely diffuse to the brain.

TJs constitute the primary structure of the paracellular barrier between endothelial cells and are responsible for regulating BBB permeability.

3. Composition of TJs at BBB

TJs of the BBB are present between the endothelial cells of brain capillaries. There are two primary classes of proteins at the TJs: i) transmembrane proteins, including occludin, claudins and junctional adhesion molecules (JAMs); ii) peripheral proteins: the zonula occludens family, AF6/afadin, multi-PDZ domain protein 1 (MUPP1), membrane-associated guanylate kinase inverted (MAGI)-1, -2 and -3, PAR-3 and -6, and heterotrimeric G-proteins.

Occludin, the first identified transmembrane TJ protein (35), is a 60-65 kDa molecule. It is characterized by four transmembrane regions, two extracellular loops, a shorter N-terminal and a longer C-terminal cytoplasmic domain. The N- and C-terminus are intracellular. The exact role of occludin in TJs remains unknown, however, the barrier function by TJs remains present in the intestinal epithelium of occludin knock-out mice, suggesting that occludin is likely a structural component of TJs. An increasing number of studies indicate that occludin is important in the formation of TJs (36,37). Occludin function is regulated by GTPases, proteases and cytokines (38).

JAMs are members of an immunoglobulin subfamily, with a molecular weight of ~40 kDa. JAM comprises three structural domains: an extracellular domain with two immunoglobulin-like loops, a single transmembrane domain and a short intracellular domain. The JAM family of proteins are divided into two groups based on their sequence similarities: the closely related JAM-A, -B and -C and the more distantly related coxackie and adenorevirus (CAR), CAR-like membrane protein, endothelial cell-selective adhesion molecule (ESAM) and JAM-4 (39). JAM-C and ESAM are involved in the promotion of melanoma lung metastasis formation (40).

Claudins are integral membrane proteins of the TJs that regulate the function of the TJs.

There are three members of the zonula occludens (ZO) family: ZO-1 (41), -2 (42) and -3. Common structural features of the ZO family include three PDZ domains in the N-terminal region, a SH3 (Src homology 3) domain and an enzymatically inactive guanylate kinase (GUK) domain. ZO proteins are important scaffold proteins, but are also essential in signaling processes (43-45). In addition, cingulin, AF-6 and 7H6 antigens are also important structural proteins of TJs. These peripheral proteins also play a role in maintaining the stability of TJs.

4. Claudins

Claudins were first identified by Furuse *et al* (41). In mammals, 24 members of the claudin-encoding gene family (CLDN genes 1-24) have been described: 23 in humans and chimpanzees and 24 in mice and rats. Claudins belong to the peripheral myelin protein (PMP22)/epithelial membrane protein (EMP) or membrane protein (MP20)/claudin superfamily of tetraspan membrane proteins (PFAM family 00822). Claudin genes are 22-34 kp in size with the majority of human claudin genes being 22-24 kp (42). The majority of claudins are located in epithelial and endothelial cells in all TJ-bearing tissues, however, a number of claudins have been observed in the cytoplasm (46,47). The claudins in the cell membranes have four transmembranal helices, with their NH_2- and COOH-terminal tails extending into the cytoplasm. A typical claudin protein contains a small intracellular cytoplasmic C-terminal sequence of ~4-5 residues, followed by a long extracellular loop of ~24 residues, a short 20-residue intracellular loop, another extracellular loop of ~24 residues and a COOH-terminal cytoplasmic tail (42,47). The amino acid sequences of the first and fourth transmembrane domains are highly conserved among claudins and the second and third are more diverse. The size of the carboxy-terminal cytoplasmic tail is most variable in length; it is typically between 21 and 63 residues. Different sections of claudin have different functions. The first extracellular loop contains highly conserved charged amino acids and is hypothesized to effect the formation of charge-selective channels in the paracellular space (48). Two highly conserved cysteines are expressed in the first extracellular loop of all claudins and potentially form an intramolecular disulfide bond to stabilize protein conformation (49). The exact function of the second extracellular loop remains unclear, however, it has been shown that claudin-5 in the second extracellular loop forms helix-turn-helix motifs with claudins on neighboring cell membranes, thus narrowing the paracellular cleft (50). The highly conserved residues Y148, Y158 and E159 in ECL2 of claudin-5 contribute to homo- and/or heterophilic trans-interaction between classic claudins and thereby tighten the paracellular space against ions, small and large molecules (51). The carboxy-terminal cytoplasmic tail of the claudins contains a PDZ-domain-binding motif that allows claudins to interact with cytoplasmic scaffolding proteins. Scaffolding proteins primarily include the TJ-associated proteins MUPP1 (52), Pals1-associated TJ protein (53), and ZO-1, -2 and -3 and membrane-associated guanylate kinase-like homologues (54). Furthermore, the COOH-terminal tail upstream of the PDZ-binding motif is required to target to the tight junctional complex and also acts as a determinant of protein stability and function (55,56).

Interactions between claudins may be homo- and heterotypic. It has been suggested that the primary structure of claudin-based paracellular pores may be formed via homotypic interactions (57,58). There are two subsets of heterotypic interactions: between claudins of the same cell membrane (side-by-side interaction) and between claudins of opposing cell membranes (head-to-head interaction). Side-by-side and head-to-head interactions are limited to specific combinations of claudins (59-62).

Claudin function is typically evaluated by sampling gene expression at a number of points corresponding to gene knock-out or overexpression in the vast majority of studies. However, knowledge of the exact function of each type of claudins remains incomplete. It has been demonstrated that claudins have a close connection with embryonic morphogenesis and abnormal expression of claudins is markedly linked to various diseases, including malignant tumours. For example, abnormal expression of claudin-1, -6 and -7 has been observed in a number of skin diseases; claudin-2, -5 and -8 are associated

with the gastrointestinal system; and abnormality in claudin-9, -11 and -14 is associated with hearing impairment (42,63).

Distribution of claudins varies in different tissues. Claudin-5 is the dominant claudin in BBB endothelial cells, although claudins-1, -3 and -12 are also expressed in these cells (64-66). Claudin-5 has been described as being the key factor involved in the endothelial permeability of the BBB (67,68).

5. Claudin-5 plays a role in the process of BM via the regulation of BBB permeability

Basic structure. Claudin-5 deficiency was first described by Morita *et al* (69) in patients with velo-cardio-facial syndrome hereditary disease. Claudin-5 is a protein encoded by the *CLDN5* gene which contains only one intron and has two transcript variants (42). It contains 218 amino acids, with a molecular weight of 23,145 Da. As it has typical molecular structure of a claudin, it is hypothesized to function as a typical claudin.

Distribution. According to Morita *et al* (69), claudin-5 is an endothelial-specific component in the brain and lung vasculatures. Morita *et al* and Rahner *et al* also demonstrated that claudin-5 is expressed in the liver ECs and dermal vascular endothelia (70,71). However, claudin-5 expression has been observed in uterine epithelial cells in the uterus of pregnant/gravid squamate reptiles and HT-29/B6 cells, an epithelial cell line derived from the human colon (72,73). It has been confirmed that the principal claudin in BBB is claudin-5 and that it is an endothelial-specific component of the cell membrane of BBB (64-66,74), suggesting that claudin-5 is important in BBB.

Function of claudin-5. Claudin-5 is observed to be a key component of the TJ strand, particularly in brain endothelial cells. The major role of claudin-5 is to selectively decrease the permeability to ions. In particular, the conserved cysteines are crucial: mutation of either cysteine eliminates the ability of claudin-5 to increase transepithelial resistance (75). The COOH-terminal tail of claudin-5 interacts with scaffolding proteins and is required for the apical localization at TJs (55). The function of TJs largely relies on homo- and heterophilic trans-interactions of claudin-5 and other claudins. Hemophilic trans-interaction is the interaction between claudin-5 proteins and heteropolymers may be formed by claudins-1, -3 and -5 (76). Claudin-3 and -5 form elliptic meshes to restrict macromolecules passing through the tissue barrier. Two populations of elliptic meshes, with a mean diameter of <100 and 300-600 nm, respectively, have been observed. This function of claudin-5 is not exclusive to BBB. It has been reported that claudin-5 expression and junctional organization controls human dermal microvascular ECs and arteriolar-capillary paracellular barriers. The barrier includes transendothelial electric resistance and macromolecular flux (77).

The role of claudin-5 in TJs has attracted attention over the past two decades. In a number of pathological processes, including inflammation, oedema, toxic damage, trauma and tumour, claudin-5 and any regulating factor have been observed to mediate the change in endothelial or epithelial permeability. It was demonstrated that matrix metalloproteinases (MMPs) open the BBB by degrading TJ proteins, claudin-5 and occludin and increasing BBB permeability following stroke. Additionally, an MMP inhibitor prevents degradation of TJ proteins and attenuates BBB disruption (78). Exposure of brain microvascular endothelial cells to high glucose increased BBB permeability in parallel with reduced expression levels of claudin-5 and also confirmed that claudin-5 is a key determinant of BBB permeability (79). Similarly, exposure to neurotoxicants malathion and lead acetate induces increased BBB permeability with decreased protein levels of TJ proteins, including claudin-5 (80). According to an *in vitro* BBB model, culture pH and buffer concentration have a significant impact on BBB permeability. This regulation may be mediated by increased claudin-5 expression (81). These data indicate that claudin-5 is important in the regulation of BBB permeability by modifying TJs.

The function of claudin-5 is regulated by a number of factors. Cyclic AMP (cAMP) was observed to elevate the barrier function of TJs in porcine BBB endothelial cells. This elevation is achieved through the protein kinase A (PKA)-induced phosphorylation of claudin-5 immuno-precipitates or via the PKA-independent induction of claudin-5 (82). Similarly, in rat lung endothelial cells, it was also observed that claudin-5 expression is required to elevate endothelial barrier functions in response to cAMP (83). ERG plays a pivotal role in regulating EC barrier function and this effect is mediated, in part, through its regulation of *CLDN5* gene expression (84). Transforming growth factor-β1 increases the tyrosine phosphorylation of VE-cadherin and claudin-5. This process is involved in the increased paracellular permeability of CNS-derived vascular endothelium (85). The correlation between claudin-5 and other cells in the BBB has also been studied. It was observed that glial cell line-derived neurotrophic factor secreted from pericytes increases the expression of claudin-5 and the TEER of brain microvascular endothelial cell, thus increasing the barrier function of the BBB (86). HIV-1 Tat protein contributes to alterations of the expression of claudin-5 and other TJs-associated proteins through activation of vascular endothelial growth factor receptor-2 and multiple redox-regulated signal transduction pathways (87,88). Other factors, including sodium caprate, β1-integrin and bradykinin are also involved in the regulation of claudin-5 (89-91).

Claudin-5 and BM. It has been hypothesized that the peritumoural brain edema in glioblastoma multiforme is a result of the downregulation of claudin-1 and -5 and occludin expression (92). One of the characteristics of a metastatic tumour is significant peritumoural edema, which may be explained as a result of the alteration in TJs and claudin-5. In superficial oesophageal squamous cell carcinoma, lymph node metastasis was observed to be associated with claudin-5 expression, although the exact mechanism is undetermined (93).

In a study of claudin-5 deficient mice, the ability of the BBB to act against small molecules (<800 Da), but not larger molecules, was selectively reduced (68). This size-selective loosening of the BBB supports the hypothesis that claudin-5 is important in selective regulation of BBB permeability, which is a determinant of tumour metastasis. Furthermore, evidence exists to support the hypothesis that claudin promotes the activation of pro-MMP-2 (94). Caveolae-dependent internalization/recycling of claudin-5 was observed to transiently increase

brain endothelial paracellular permeability during CNS inflammation (95,96). In addition, claudin-5 was demonstrated to regulate endothelial motility. Escudero-Esparza *et al* (97) inserted claudin-5 into a human vascular endothelial cell line and noted a significant downregulation of motility, adhesive to matrix and angiogenic potential of vascular endothelial cells, indicating that claudin-5 may function through N-WASP and ROCK signaling pathways. A similar phenomenon was observed in breast cancer. Findings of those studies suggest that claudin-5 alters the biological behavior of tumour cells and plays a role in the formation of tumour metastases. In addition, it was observed in human immunodeficiency virus-1 encephalitis, that Rho kinase directly induces phosphorylation of occludin and claudin-5, resulting in decreased barrier tightness and enhanced monocyte migration across the BBB (98,99). This mechanism may provide an explanation as to the increased permeability of BBB and cell migration across the BBB. Whether brain metastases follow the same mechanism remains unknown.

6. Conclusion and future prospects

Intracranial metastatic tumours have a higher incidence compared with primary intracranial tumours. BM involves a number of steps. A vital step is the transmigration of detached primary tumour cells through the BBB into the brain parenchyma. The BBB comprises endothelial cells, perticytes and astrocytes. These components play various roles in the BBB, making the BBB a selective barrier. TJs constitute the main structure of the paracellular barrier between endothelial cells and claudins and are key proteins regulating permeability of TJs. The principle claudin in BBB is claudin-5. This claudin has two primary functions: regulation of BBB permeability and regulation of cell motility. These two functions are involved in the mechanism of brain metastases. Therefore, the association between claudin-5 and brain metastases is of great interest. Two issues remain to be resolved: how the primary tumour cells affect the vital claudins (claudin-5) in the TJs of brain microvascular endothelial cells and result in increased BBB permeability and BM and through which mechanism the claudins (claudin-5) regulate the motility, adhesion to the matrix and angiogenic potential of the tumour cells to complete their transportation.

The role of the BBB in brain metastases has attracted great interest. A number of studies have been performed on transmembrane proteins, including claudins and occludins. However, the majority of studies focus on the correlation between the changes in TJ proteins in carcinoma cells and enhancement of invasion ability of the tumour. A number of investigators have considered the interaction between tumour cells and endothelial cells. However, the endothelial cell lines used, such as HECV, in these studies are derived from the umbilical vein system, not from brain microvascular endothelial cell lines, thus they do not represent ECs in the BBB. Claudin-5, as a distinct and important transmembrane protein of BBB TJs, is confirmed to be involved in the process of tumour cell migration into the brain through paracellular passage. However, little is currently known of the regulation of claudin-5 on BBB TJs and the signaling pathways involved in BM. It is clear that this area requires further investigation.

Acknowledgements

The authors would like to thank Cancer Research Wales and the Albert Hung Foundation for supporting their study. Dr W Jia is a recipient of the China Medical Scholarship of Cardiff University.

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