Junctional Adhesion Molecules in Cerebral Endothelial Tight Junction and Brain Metastasis

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Abstract. *Brain metastasis is one of the most deadly types of metastasis, frequently seen as a result of cancer spread from lung cancer, breast cancer and malignant melanoma. A key cellular structure in controlling brain metastasis is the blood–brain barrier (BBB). The BBB is known to protect metastatic tumour cells from chemotherapy and antitumor immunity. On the other hand, the BBB is also a key cellular structure which cancer cells must breach before settling in brain tissues. Tight junctions (TJs), central to the BBB, have received much attention in recent decades. There has been progress in investigating cerebral TJs and brain microvascular endothelial cells. Junctional adhesion molecules (JAMs) are transmembrane proteins within TJs and have been shown to be key to the integrity of the BBB and to play a role in controlling brain metastasis. The current article summarizes the recent progress in the regulation of JAMs in BBB TJs and the signaling pathways involved during brain metastasis.*

Brain metastases (BM) are the most dreaded complications of systemic cancer. The most common primary tumour sites for brain metastasis are the lung (40%-50%), breast (15%-25%) and skin (5%-20%) (1). Taking lung cancer as an example, 20% of patients with small-cell lung carcinoma (SCLC) have brain metastases at diagnosis of the primary tumour and up to 80% have central nervous system (CNS) metastases at autopsy. During the course of non-small cell lung carcinoma (NSCLC), approximately 30% of patients will have metastatic brain tumours, most of which are large-cell undifferentiated carcinomas and adenocarcinomas, followed by squamous carcinomas (2, 3).

With more readily available and more effective treatment for primary tumours, patients with cancer are living longer than ever before. As a result, patients are likely to develop distant metastasis , and with improved imaging techniques, more patients are diagnosed with metastasis, particularly at an early stage. Treatment for BM includes surgery, radiotherapy and chemotherapy, performed singly or in combination, according to the actual situation. Although advances in medical technology have significantly improved patient survival, the mechanisms by which BM develop remains unclear. Although most theories of metastasis, including the seed and soil hypothesis (4, 5), the stress and mast cell activation theory (6, 7), the blood–brain barrier theory (1), and stem cell theory (8), are possibly applicable, it is perhaps the BBB that is a more attractive argument when one discusses BM, efficacy of chemotherapy and other types of therapies for BM.

Metastatic spread of cancer is commonly performed *via* blood vessels or lymphatic vessels. However, for BM the only route is through the blood vessels, owing to the lack of lymphatics in the CNS. Rich anastomoses exist between the intracranial vascular networks and ascending cervical arteries, therefore, lung cancer cells can bypass the pulmonary capillary filtration and directly invade the CNS, resulting in a significantly higher incidence of BM than that from other primary tumours. Regardless of their source, however, metastatic tumour cells have to transmigrate through the BBB to invade the brain.

The BBB is an important structure which provides a stable microenvironment for the CNS. Complicated neural functions rely on the integrity of the BBB, which is a complex of non-interspace endothelial cells, TJs , basement membrane, pericytes and astrocyte end-feet (9). CNS diseases often cause dramatic changes in the structure and function of the BBB. For example, neonatal jaundice and vasogenic cerebral edema, induce the opening of the

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Key Words: Junctional adhesion molecules (JAMs), brain metastasis blood–brain barrier (BBB), Tight junctions (TJs), review.

capillary endothelial TJs, and significantly increase the permeability of the CNS barrier, so that macromolecules such as albumin (69 kDa) can pass through the barrier; serious injury causes severe damage of the BBB, allowing serum proteins to diffuse through the barrier into the brain. During the healing process, initially macromolecules then micromolecules are blocked outside the brain and finally, when it heals, free diffusion is prevented once again. In addition, ionizing radiation, laser and ultrasound can increase the permeability of BBB.

Brain capillary endothelial cells and endothelial TJs are essential structures of the BBB (10). The TJs are mainly composed of transmembrane proteins, cytoplasmic attachment proteins and cytoskeletal proteins. Junctional adhesion molecules (JAMs), a group of transmembrane proteins, are involved in cell-to-cell or cell–extracellular matrix binding, and expression of JAM-1 directly affects TJ functions. The BBB not only hinders solute transport, but also restricts the free movement of cellular elements between the systemic circulation and neuronal tissue. Surprisingly, some types of tumour cells can pass through the barrier, while most of other cells are prevented from entering the brain. This raises the following questions: How does the BBB interact with metastatic cancer cells? What are the conditions necessary for the selective opening of the barrier to tumour cells? Are there chemokines that attract tumour cells to the brain tissues by passing through the BBB?

Disruption of the TJs of the BBB is an important link in the development of brain metastasis (11). It has been found that transmembrane transporters (*e.g.* JAM family proteins) expressed at the TJs may serve as key negative regulators of cancer cell invasion and possibly metastasis (12, 13). Huber *et al.* suggested that brain vascular endothelial cells can actively participate in metastasis, promoting an increase in paracellular permeability or even providing an ideal living environment for metastatic tumour cells (11). These metastatic cancer cells may be protected by the BBB from immune surveillance; meanwhile, substances released by the BBB may favour the growth of tumour cells. This article reviews the recent progress in knowledge on JAMs and focuses on the molecular mechanisms by which BBB TJs are damaged during the process of brain metastasis.

The JAM Family: Structural Features, Expression and Location

Members of the JAM family. JAMs are transmembrane proteins belonging to the cortical thymocyte marker of the Xenopus (CTX) family, a member of the immunoglobulin (Ig) superfamily. Major members of the JAM family include: JAM-A (JAM-1, F11R or the 106 antigen), mainly expressed in endothelial and epithelial cells (14, 27, 29, 52, 60, 61); JAM-B (JAM-2, VE-JAM, hJAM-2 or mJAM-3) (15, 16, 26, 62); and JAM-C (JAM-3, hJAM-3 or mJAMM-2) (16, 22, 43, 63). Other JAM family members include JAM-4 (17) and JAM-L (AMICA1) (18) (Table I). In addition, the endothelial cell-selective adhesion molecule (ESAM) (19) and the Coxsackie and adenovirus receptor (CXADR or CAR) (20) are structurally related and share homology with the JAMs. However, the classic JAM family only includes three members: JAM-1, JAM-2 and JAM-3. At the protein level, they display a high (32%-38%) homology to each other (21). The main difference between classic JAMs and the related proteins is the length of cytoplasmic domain: classic JAMs have a short 40-50 amino acid tail, whilst the related proteins have a long 105-120 residue tail.

Table I. *The nomenclature of the junctional adhesion molecule(JAM) family.*

Name	Species	Designation	Reference
JAM-A	Mouse	106 antigen	(60)
	Mouse	JAM	(52)
	Human	JAM	(61)
	Human	JAM	(29)
	Human	JAM-1	(27)
JAM-B	Human, mouse	VE-JAM	(15)
	Human	JAM2	(26)
	mouse	JAM-3	(62)
	Human	VE-JAM/JAM-2	(16)
JAM-C	Human	JAM-3	(22)
	Mouse	JAM-2	(63)
	Human	JAM-3	(43)
JAM4	Mouse	JAM-4	(17)
JAML	Human, bovine	JAML	(18)

Structural features of JAMs. The JAMs are composed of an extracellular domain, a single transmembrane segment and a cytoplasmic tail of variable length. All these molecules contain Ig-like domain(s) in their extracellular domain. The Ig-like domains can be divided into four groups: C1, C2, V and I types. All JAMs have two extracellular Ig domains. JAM-2 and JAM-3 are structurally related and share a similar organization: a membrane-distal V-type Ig-domain and a membrane-proximal C2-type Ig-domain (22). The polypeptide chain of the extracellular region of human JAM-1 folds into two concatenated Ig-like domains, a V-set and an I-set (23).

Like other adhesion molecules, JAMs also contain a single transmembrane segment and a short cytoplasmic tail. The cytoplasmic tail is about 40 residues in length, and ends in a special motif (Phe-Leu-Val) (24) for binding proteins containing (PDZ) domains. JAM-1 contains one single disulfide bond in each extracellular Ig-like domain, whereas JAM-2 and JAM-3 have two for each, this may partly explain why JAM-2 and JAM-3 have stronger structural stability than

JAM-1 (25). There is a potential phosphorylation site in the short intracellular tail, which can be phosphorylated by protein kinase C (PKC), protein kinase A (PKA) and casein kinase II (22, 26-27). Mutation of serine 281 in the cytoplasmic tail of JAM-3 abolishes the specific localization of JAM-C in TJs and the establishment of cell polarity and further stimulates integrin-mediated cell migration and adhesion *via* the modulation of integrin activation (28). The PDZ-binding domain at the end of *C*-terminal may be associated with signal transduction and is a possible binding site for intracellular molecules. In addition, the extracellular regions of JAMs also contain an N-glycosylation site (15, 26) with unclear physiological functions.

Expression and location of JAMs. JAM-1 is expressed on the surface of endothelial and epithelial cells of multiple organs and tissues, including the liver, kidney, pancreas, heart, brain, lymph nodes, intestines, lungs, placenta and vascular tissues. Moreover, expression of JAM-1 has also been found in platelets, monocytes, lymphocytes, neutrophils and antigen-presenting cells such as macrophages (29). However, the pattern of expression of JAM-2 and JAM-3 is more exclusive to endothelial cells, particularly in the high endothelial venules (HEV) and lymphatic ducts (15). JAM-2 is not expressed in leukocytes, while human JAM-3 is expressed in platelets, monocytes, natural killer cells, dendritic cells, B-lymphocytes and some T-lymphocytes (16). JAMs in endothelial and epithelial cells localize at cell–cell contact, and regulate TJ formation in these cells. In addition, JAMs may be associated with intracellular communication and cell polarity-related proteins may be recruited by the PDZ-binding domains of JAMs. It has been shown that JAM-1 plays an important role in the regulation of TJ assembly in epithelia (29): skeleton proteins containing a PDZ domain closely interact with other transmembrane proteins, such as claudin and occludin, to form a large TJ complex (30). In 1999, *in vitro* and *in vivo* studies by Del MA *et al*. demonstrated that a blocking monoclonal antibody (BV11 mAb) directed to JAM-1 significantly inhibited leukocyte infiltration in the brain parenchyma (31). The association between JAM-2/-3 and (ZO1) and (PARD-3) only appears in endothelial cells, however, it plays an important role in leukocyte recruitment during inflammation (32).

How do JAM proteins work in TJs? As adhesion molecules, JAMs participate in many physiological processes related to cell adhesion. Similar to most adhesion molecules of the Ig superfamily, JAMs are involved in both homophilic and heterophilic interactions.

Homophilic interaction. JAM-1 proteins from the same cell form U-shaped homodimers mediated through a dimerization motif (Arg-Val-Glu) that is present in the N-terminal Ig-like domains. Homophilic interaction is crucial for JAM-1 function: Pairs of cis- dimers from adjacent cells contact each other *via* their V-type Ig-domains to link adjacent cells (33). This homophilic adhesion widely exists in JAM-1, JAM-2 (Arg-Leu-Glu) and JAM-3 (Arg-Ile-Glu).

When expressed in Chinese hamster ovary (CHO) cells or Madin-Darby canine kidney (MDCK) cells, JAMs localize only to the cell–cell junctions formed by two transfected cells and not to those formed by co-work of a transfected cell with an untransfected cell (27). Research on recombinant soluble JAM-1 (rsJAM-1) also demonstrated this type of homophilic interaction in JAM-1: rsJAM-1 proteins were cultured with CHO cells expressing JAM-1; analysis of rsJAM by equilibrium centrifugation revealed that the molecular mass of the JAM-1 complex on the CHO cell surface doubled (34). These data confirm the existence of cell-to-cell homophilic interactions. Adhesive force is exerted by homophilic interactions between the JAM-1 of platelets and endothelial cells (35). Protein binding assay of recombinant JAM2-Fc demonstrated that JAM-2 is capable of homotypic interactions (26). Homophilic interaction of JAM-3 has also been confirmed (36). An epitope including mutational residues within the putative homodimer interface was shown to block JAM-1 homodimer formation and prevent enrichment of JAM-1 at the points of cell contact (37). Homophilic interactions of JAMs greatly affect the endothelial and epithelial permeability, thus playing an important role in inflammatory responses (38).

Heterophilic interaction. Besides homophilic interactions, heterotypic interactions also exist in JAM proteins. It has been reported that JAM-2 interacts with JAM-3 in a heterophilic manner (16, 22, 26, 39). An *in vitro* study demonstrated that JAM-2 may dissociate soluble JAM-3/JAM-3 homodimers to form JAM-2/JAM-3 heterodimers. This suggests that the affinity of JAM-2 monomers in forming dimers is higher for JAM-2 than for JAM-3 (40). Evidence shows that JAM-2 adheres to T-cells, natural killer cells and dendritic cells through interactions with JAM-3 (16). Heterophilic interactions also exist between JAMs and other types of cell adhesion molecules, such as integrin. It has been demonstrated that JAM-1 contributes to integrin lymphocyte function-associated antigen 1 (LFA-1 or CD11c/CDl8)–dependent transendothelial migration of neutrophils through endothelial monolayers; JAM-1 supported LFA-1-mediated adhesion of leukocytes to endothelia when JAM-1 localized to the top of endothelial cells (41). JAM-2 appears to contribute to leukocyte extravasation by facilitating transmigration through interaction with $\alpha_4\beta_1$ integrin (39, 42). In addition, JAM-3 can adhere to CD11b/CDl8 (43).

Role of JAMs in assembly and stability of cell junctions. JAMs, especially JAM-1, play an important role in the regulation of TJ assembly. Evidence for this is as follows.

These proteins have been reported to reduce cell permeability and increase resistance to macromolecules. In an inflammation model, antibodies to JAM-1 increased vascular endothelial permeability (29); JAM-1-specific mAbs or soluble JAM-1 markedly inhibited transepithelial resistance recovery by transient calcium depletion (16, 29).

Expression of JAMs is usually proportional to the number of TJs formed. Moreover, JAM-1 appears earlier than other marker molecules of junctions during the formation of cell–cell contacts (44). The possible process may be: JAM-1 is initially recruited to the nectin-based cell–cell adhesion sites, then it adheres to ZO-1 to form the TJ complex (45). In addition, overexpression of mutant JAM-1 disrupts calcium/calmodulin-dependent serine protein kinase (CASK) recruiment at intercellular TJ (30).

JAM-1 has a PLV sequence (*N*-terminal residues 298-300) for binding proteins containing type II PDZ domain. JAM-1 can bind to five PDZ proteins: ZO-1, Afadin-6 (AF6), CASK, Partitioning defective 3 homologue (PAR3) and Multiple PDZ Domain Protein-1 (MUPP1) (30, 44, 46, 47). These proteins are essential for the assembly of TJs because formation of actin skeleton and modulation of cell polarity depend on them. *In vitro* studies have demonstrated that direct interactions between JAM-1 and the PDZ– domain of these proteins play a role in several steps of junction assembly (48). JAM-2 and JAM-3 also have a potential PDZ–domain-binding ability; however, their exact roles in TJs remain unclear.

Relationship between JAMs and BMs. JAM, which mediates homophilic adhesion, is an important component of endothelial TJ complex. JAM-1 is robustly expressed in tissues rich in vascular bed and lymphatic ducts, such as normal human mammary epithelium, and its expression is down-regulated in metastatic breast cancer and squamous cell carcinoma (49). JAM may therefore be involved in the adhesion between cancer cells. Homophilic interactions tend to occur between JAM-1 proteins (34). During the course of tumour development, cells in normal tissues are gradually replaced by cancer cells, which means that cancer cells and normal cells gradually become exogenic cells. Thus, homophilic adhesion is weakened, leading to a loose connection between the tumour cells and the primary lesion, in favour of the detachment of tumour cells. Detached tumour cells adhere to and degrade extracellular matrix and subsequently migrate to a distant site. Gutwein *et al*. induced metastasis of renal cell carcinoma through down-regulation of JAM-1 expression by using siRNA interference (50). It is reasonable to believe that JAM-A down regulation is an early event in the development of renal cancer and increases the migration of renal cancer cells. Hepatocyte growth factor (HGF) is a cytokine involved in tumour metastasis. Martin *et al*. demonstrated that HGF disrupts TJ function in human breast cancer cells by causing changes in the expression of TJ molecules (occludin, claudin-1, claudin-5, JAM-1 and JAM-2) at both the mRNA and protein levels, resulting in reduced trans-epithelial resistance and increased paracellular permeability of the cells (51). These findings, thus, suggest that JAM-1 is expressed both in normal tissues and tumour cells. With the progression of cancer, JAM-1 expression declines, and formation of the TJ complex decreases or even disappears, leading to TJ disconnection, which results in the tumour cells detaching from the primary lesion. The opening of epithelial TJs of microvasculature and lymph ducts around the tumour is also increased, creating conditions for distant metastasis.

Relationship between JAM and the Integrity of the BBB. The BBB is a complex system composed of different cells, including endothelial cells, astrocytes, pericytes and perivascular mast cells. TJs of the BBB control paracellular permeability to circulating cells and solutes, preventing the CNS from outside toxic damage. JAMs are indispensable for brain endothelial TJs (52, 53) and JAM-1 plays an important role in the initial stage of TJ assembly, which is essential for the integrity of the BBB.

JAM proteins are able to reduce cell permeability and increase resistance to macromolecules. In an inflammation model, a JAM-1 antibody was shown to increase vascular endothelial permeability (29). The expression of JAMs is usually proportional to the number of TJs. JAM-1 is expressed earlier than other marker molecules of junction in cell–cell contact (44). JAM-1 can bind to 5 PDZ proteins, ZO1, AF6, CASK, PAR3 and MUPP1 (30, 44, 46, 47). These proteins are quite important for the assembly of TJs because formation of actin skeleton and modulation of cell polarity depend on them. An *in vitro* study has demonstrated that direct interactions between JAM-1 and the PDZ–domain of these proteins play a role in several steps of junction assembly (48). JAM-2 and JAM-3 also have a potential PDZ–domain- binding ability.

Altered expression of these TJ proteins could cause BBB breakdown following brain injury leading to edema. This is supported by a significant decrease in JAM-1 expression noted in the lesion site after brain damage (54). All these studies point to an important role of JAMs in the regulation of BBB TJ function.

Similarity between BM and leukocyte extravasation in inflammatory response. Tumour cells transmigrate across the endothelia through two major routes: the paracellular route (*via* cell–cell junctions) and the transcellular route (*via* individual endothelial cells) (55, 56). Paracellular migration of cancer cells involves endothelial TJs and adherens junctions. According to a recent study reported by Fazakas *et al*., different types of melanoma cells reduced transendothelial

electrical resistance (TEER), a widely used indicator of the integrity of endothelial junctions, in an *in vitro* BBB model (57). In addition, melanoma-conditioned media also induced a less pronounced decrease in TEER. An immunofluorescence study demonstrated that down-regulation of claudin-5 and ZO-1 increases TJ permeability. Melanoma cells were found to form holes in the endothelial cell monolayers when they were co-cultured for a long time; other metastatic tumour cells were also found to have this ability. The exact mechanism of how metastatic cells destroy TJs remains to be elucidated. Proteolysis may play an important role here. On the other hand, it cannot be excluded that tumour cells may also transmigrate through a transcellular route, especially the sealed intercellular transport route between brain endothelial TJs. Transendothelial migration *via* the transcellular route has so far only been seen in an artificial vascular network (calf pulmonary artery endothelial cells source) invaded by breast cancer cells (58).

Leukocyte infiltration is a consequence of the interaction between leukocytes and activated endothelial cell monolayers. This is a sequential process of three steps: localization, adhesion and transmigration through the endothelial layer (59). Adhesive interactions between ligand expressed by leukocytes and receptor expressed by endothelia are involved in these steps. Similarly, metastasis of tumour cells to brain includes several consecutive steps: apposition, attachment and penetration. These steps require effective interactions between the BBB and tumour cells, and adhesion molecules play an role in these interactions. In the apposition period of metastasis or leukocyte adhesion, the tumour cell–BBB or leukocyte–endothelium molecular dialogue requires the contribution of cytokines, chemokines and other signal transfers. The tumour cells are guided to the metastatic foci just like the leukocytes are guided to the inflamed area. During the attachment period, specific ligand–receptor interactions play a vital role in both pathophysiological procedures. Finally, in the penetration period, tumour cells transmigrate through the BBB into the brain, whereas leukocytes infiltrate into inflamed areas.

Perspectives

Brain metastasis is closely related to damage of the BBB; the BBB can protect metastatic tumour cells against chemotherapy and anti–tumour immunity. Therefore targeted therapy inhibiting the transmigration of tumour cells through the BBB may be a promising treatment method. The similarity between leukocyte extravasation and BM provides a new research direction in studying the mechanism of tumour cell migration through the BBB.

Given the rarity of endothelial cells and endothelial cell lines derived from brain tissues, it is inevitable that a number of the arguments stated in the current article are inferred and extrapolated from studies on endothelial cells of other tissues and other species, and indeed other cell types such as leukocytes. It is nonetheless noteworthy that recent years have seen the establishment of human brain endothelial cell lines, for example hMEC/D3 as a model of the human BBB (64, 65). Such models will be essential resources for future investigations of human BBB and its role in cancer metastasis.

The role of the BBB in BM has aroused great research interest. However few studies have involved the interactions between tumour cells and brain microvascular endothelial cell. JAMs play an important role in controlling BM by maintaining the integrity of the BBB. The regulation of JAMs on TJs of the BBB and signaling pathway during BM remains unclear and is a fertile area to explore in future studies.

Acknowledgements

The Authors wish to thank Cancer Research Wales and the Albert Hung Foundation for supporting their work.

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Received April 24, 2013
Revised May 14, 2013
Accepted May 15, 2013