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Review

Conflicting roles of Connexin43 in tumor-induced inflammation and cancer pathology

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Abstract: The tumor microenvironment is known to have increased levels of cytokines and metabolites, such as glutamate, due to their release from the surrounding cells. A normal cell around the tumor that responds to the inflammatory environment is likely to be subsequently altered. We will discuss how these abnormalities will support tumor survival via the actions of gap junctions (GJs) and hemichannels (HCs) which are composed of hexamer of connexin 43 (Cx43) protein. In particular, we will discuss how GJ intercellular communication (GJIC) in glioma cells, the primary brain tumor, is a regulatory factor and its attenuation leads to tumor invasion. In contrast, the astrocytes, which are normal cells around the glioma, are "hijacked" by tumor cells, either by receiving the transmission of malignant substances from the cancer cells via GJIC, or perhaps via astrocytic HC activity through the paracrine signaling which enable the delivery of these substances to the distal astrocytes. This astrocytic signaling would promote tumor expansion in the brain. In addition, brain metastasis from peripheral tissues has also been known to be facilitated by GJs formed between cerebral vascular endothelial cells and cancer cells. Astrocytes and microglia are generally thought to eliminate cancer cells at the blood brain barrier. In contrast, some reports suggest they facilitate tumor progression as tumor cells take advantage of the normal functions of astrocytes that support the survival of the neurons by exchanging nutrients and metabolites. In summary, GJIC is essential for the normal physiological function of growth and allowing the diffusion of physiological substances. Therefore, whether GJIC is cancer promoting or suppressing may be dependent by what permeates through GJs, when it is active, and to which cells. The nature of GJs, which has been ambiguous in brain tumor progression, will need to be re-visited and understood together with new findings on Cx proteins and HC activities.

Keywords: connexin43; central nervous system; glioma; astrocyte; blood brain barrier; neurovascular unit

1. Introduction

The gap junction (GJ) protein connexin (Cx) 43 forms intercellular channels permitting the passage of small ions and signaling molecules between adjacent cells (1, 2). In particular, connexin43 (Cx43), an ubiquitous isoform, is deeply involved in regulating cell functions mainly via three kinds of approaches; one is the channel function of the GJ as an intercellular junction, second is the 'hemichannel (HC)' permitting paracrine communication between the cytosol and the extracellular environment (3, 4), and third is the non-channel function via its C-terminal where intermolecular interaction occurs.

The oncogenicity of a tumor cell depends on its proliferative capacity, motility and viability. Due to the nature of Cx that functions at the cell surface, Cx is associated with all of these cellular phenotypes. Induction of inflammatory signaling, such as the treatment of anti-cancer agents and exposure to cytokines, also modulates Cx functions, intra- and extra-cellularly. Therefore, Cx can be

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a target of drug development, however, whether they promote exacerbations or support clinical improvement depends on the surrounding environment: which cell (molecule) the Cx is communicating with, or by which structure Cx exerts its function.

The pivotal role of Cx43 in promoting tumor progression has been previously overlooked because downregulation of Cx43-mediated intercellular communication is normally associated with increased malignancy in tumor cells (5, 6). Indeed, for a long period, it was believed that the GJ formed by Cx43 has mostly tumor suppressive effects (5-7), such as its role in anti-proliferation (8, 9), anti-metastasis (10), and pro-apoptosis (11-15). In contrast, emerging evidence indicates that Cx43 serves a facilitative role in tumorigenesis (5, 16), especially in advanced stages (7). Additionally, it has been demonstrated that Cx43 allows the tumor cells to hijack programs that are part of normal tissue development (17).

In this review, we will focus on the paradoxically unique characteristics of Cx43 in cancer pathology of the central nervous system (CNS), in particular, the gliomas which are aggressive brain tumors, and metastatic cancers which move from the peripheral tissues into the brain, with reference to our recent findings.

2. the Role of Cx43 in Tumors Of The CNS

In the CNS, several Cx molecules have been observed; Cx30, Cx32, Cx36 and Cx43 in neurons, Cx30, Cx40, Cx43, and Cx45 in astrocytes, Cx32, Cx36 and Cx43 in microglia, Cx26, Cx32, Cx29, Cx36, and Cx47 in an oligodendrocyte (18-20). Although they were observations from rodents, Cx43 is one of the subtypes that showed high conservation in the majority of positions shown to be important residues for channel oligomerization, gating, permeation and docking (21) and is the major subtype in the CNS. GJs formed by Cx43 are often observed between astrocytes, and cell junctions with heterologous cells have also been reported. The paradoxical roles of this molecule are highlighted in Table 1 which highlights three types of formations as GJs, HCs, or non-channel functions (inter-molecular interaction).

Table 1. Summary of the role of Cx43 with specific cell combinations in cancer pathology.

Cell combination	Form of Cx	Transmitter or Partner molecule	Effect on malignant behavior and mechanisms suggested to be occurred in cancer cells	Ref
Mesothelioma-Mesothelioma	Cx molecule (C-terminal)	Src, Bax, JNK	Increasing level of Cx43 in malignant mesothelioma cell **enhances sensitivity** against cisplatin and sunitinib treatment	[11], [12], [13],
Leukemic cell-BMSCs	GJ	-	GJ between Cx43- overexpressed BMSCs and leukemic cells **induced apoptosis in leukemic cells** due to caspase 3/7 activation	[14], [15]
Breast cancer-Osteocyte	HCs	ATP	Released ATP from osteocyte **inhibits growth, migration and invasion ability of breast cancer cells**	[9]
Glioblastoma-HMEC	GJ	miR-145-5b	miR-145-5b from HMEC is transferred to glioblastoma (U87 cells) which **decrease cancer proliferation**	[104]
Colon cancer-HMEC	GJ	miR-145-5b	miR-145-5b from HMEC is permitted to be transferred to cancer cells (SW480 cells) and up-regulated Cx43 expression, which **inhibits proangiogenic** effect of cancer cells	[116]
Glioma-Glioma	GJ, Cx molecule (extracellular loop and/or C-terminal)	miR-5096	GJ between glioma-glioma has **anti-invasive** effect	[22], [72]

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Glioblastoma-HMEC	GJ	miR-5096	mir-5096 from glioblastoma is transferred to HMEC **increases proangiogenic** effect of glioblastoma	[104]
Glioma-Glioma	GJ, Cx molecule (C-terminal)	Bcl-2, Bax	Increasing level of Cx43 in glioma cell **enhances resistance** against temozolomide treatmentIncreasing level of Cx43 in glioma cell **enhances resistance** against temozolomide treatment	[86]
Glioma-Astrocyte, Astrocyte-Astrocyte	GJ, Cx molecule (extracellular loop and/or C-terminal)	miR-5096	Glioma-astrocyte and astrocyte-astrocyte **promotes glioma invasion**	[22], [72]
Glioblastoma-HMEC	GJ	miR-5096	mir-5096 from glioblastoma (U-87 cells) to HMEC **increases proangiogenic** effect	[104]
Melanoma-Astrocyte	GJ	-	Direct contact with astrocyte **up-regulates invasion** of cancer cells and **drug resistance**	[126]
Breast cancer-Astrocyte, Lung cancer-Astrocyte	GJ		GJ signaling enhances production of **cytokines in cancer cells** and **endothelin in astrocytes**, which in turn **upregulate AKT/MAPK signaling in breast cancer (MDA-MB-231) and lung cancer (H226) cells** to protect from cytotoxicity of chemotherapeutic drugs	[127]
Breast cancer-Astrocyte, Lung cancer-Astrocyte	GJ	cGAMP	**cGAMP from metastasized cancer cells** to astrocytes induces **STING signaling in astrocytes**, which in turn **stimulate cancer metastasis**	[129]
Microglia-Retinal cerebral endothelial cell	HCs		Microgila secretes basigin via activation of PI3K/Akt signaling or IGF signaling that in turn **promote angiogenesis in cerebral endothelial cell**	[117]
Microglia-Astrocyte	GJ, HCs	IL-1β, TNF-α	Intercellular diffusion of glucose in CNS via GJ composed of Cx43 between astrocytes **is downregulated by cytokines secreted from HCs of microglia**. Opposingly when glucose uptake in each astrocyte is **increased**, then it switches the cell to be a reactive astrocyte.	[3]

Tumor suppressive effect of Cx43 is in green while those in pink indicates Cx43 works in a tumor promotive manner. Although Table 1 contains Cx43 reports regarding various cell combinations not limited to CNS, it is generally accepted that the fundamental role of connexin is the same in peripheral tissues and the CNS. BMSCs; bone mallow stroma cells, cGAMP;2'3'-cyclic GMP-AMP, CNS; central nervous system, DC; dendritic cells, GJ; gap junction, HCs; hemichannels, HMEC; human micro vascular cerebral endothelial cells.

2.1. Cx43 in Glioma Progression

The role of Cx43 protein in glioma cells in growth control and migration remains unclear despite extensive studies (5, 16). Carcinogenesis is usually accompanied with a reduction in GJIC (5, 6). We investigated Cx43 expression at both the mRNA and the protein level (16); consistent with previous studies, we have observed a general reduction of Cx43 protein in the tumor core as the glioma grade increases (22). Interestingly, Cx43 staining is increased in low grade gliomas. A query on the provisional TCGA revealed upregulation of Cx43 in low-grade gliomas may be correlated with a shorter disease-free period. These results reveal a possible role of Cx43 in the early stages of glioma

progression when the relatively low proliferative index of cells may predispose them to migrate and integrate with the host parenchyma.

2.2. Cx43 Activity in Glioma-Induced Inflammation

Inflammation is associated with cancer initiation and progression (23). The inflammatory response induced in the host tissue by the tumor cells accounts for the major signaling pathways hijacked by tumor cells to promote their own survival and expansion (24). In the brain, the release of ATP also leads to the activation of purinergic receptors in microglia, which are the resident immune cells and a prominent feature of glioma pathology (24). Microglia have a critical role in modulating the microenvironment by secreting cytokines that affect tumor growth and migration (25). However, their role in gliomagenesis remains undefined since their activation can result in pro- and anti-inflammatory responses (26, 27), therefore suppression or depletion of microglia have been demonstrated both to reduce (28, 29) or promote glioma invasion (30).

Opening of unpaired Cx43 HCs (31-33) under pathophysiological conditions results in the release of bioactive molecules including ATP (34) and participates in pro-inflammatory responses. Similarly, the pannexin (Panx) family of GJs generally form the equivalent of a HC providing direct communication between the cytoplasm and extracellular space (31, 35). Located at the plasma membrane, Panx1 is a major conduit for ATP during apoptosis that increases extracellular ATP resulting in neuroinflammation (36-38). In this regard, the association of Panx1 with P_2X_7 purinergic receptor in an inflammasome containing caspase 1 is necessary for its ATP release (39-41). Interestingly, there is evidence to suggest cross talk between Cx43 HCs and Panx1 (42) and HC activity can propagate apoptosis in glioma cells (43). Therefore, there is a possibility that inhibition of HC-mediated ATP release will reduce the recruitment of microglia and affect glioma progression.

2.3. Reactive Astrocytes, Immune Cells, and Cx43 Expression in the Glioma Microenvironment

The interaction between tumor cells and their microenvironment is implicated in modulating tumor progression and contributing to the resistance of tumor cells to therapeutic treatment (44-49). An early study indicates that 90% of recurrent gliomas occur within 2 cm of the resected tumor (50), the peri-tumoral region that is widely known as the invasive niche populated with activated immune cells, reactive astrocytes and cerebral endothelial cells. Indeed, one prominent feature of glioma pathology is extensive astrogliosis, an inflammatory response that involves the recruitment of reactive astrocytes around gliomas and brain metastases (51-53); it is often clinically difficult to distinguish glioma cells from reactive astrocytes (51). Reactive astrocytes express increased levels of glial fibrillary acidic protein (GFAP) intermediate filaments (54, 55) and pro-inflammatory cytokines (56). Although many signaling proteins are upregulated in activated astrocytes, the significance of these proteins, and whether they can be exploited to control brain diseases, remains to be explored.

The gap junction protein Cx43, widely expressed in adult astrocytes (57, 58), has been shown to be upregulated in regions with astrogliosis induced by various brain pathologies including brain ischemia and epilepsy (18, 59-65). Using a mouse model consisting of intracranial syngeneic implantation of mouse GL261 glioma cells, we demonstrate that reactive astrocytes with enhanced GFAP expression show an upregulation of Cx43 (66).

2.4. opposite Roles of Cx43 in Tumor Invasion and Apoptosis

Over-expression of Cx43 has been reported to promote glioma migration in a channel-dependent manner (67-69), especially in the presence of normal stromal cells such as astrocytes (67-70). Cx43-mediated GJIC appears to alter astrocyte morphology in an *in vitro* co-culture of glioma cells with astrocytes (69, 71), suggesting that bi-directional signaling exists between these cells. However, it remains unclear how these interactions modify the invasive properties of glioma cells *in vivo*. We similarly showed that co-culture of U87MG human glioma cells with normal human astrocytes enhances the invasive behavior of the glioma cells (72). We further demonstrated using chemical inhibitors, siRNAs, and a channel defective Cx43 mutant Cx43T154A, that functional glioma-glioma

GJs suppress glioma invasion while glioma-astrocyte and astrocyte-astrocyte GJs promote glioma invasion (72). Therefore, migration and invasion are enhanced with low homocellular glioma GJIC and high heterocellular glioma-astrocyte GJIC. Cx43 is also known to promote migration through channel-independent mechanisms; it strengthens adhesive connections between glioma cells and astrocytes via its extracellular loops (67, 68, 73, 74), and also regulates cytoskeletal dynamics via its carboxy (C)-terminal tail (75-77). The C-tail of Cx43 interacts with various intracellular signaling molecules (12, 76, 78, 79) and serves a role in channel gating (60, 80, 81).

In addition to glioma, there is also metastatic cancer cells in CNS coming from outside the brain (82). After metastatic cells have survived the harsh blood flow environment, they still have to go beyond several layers of cells to further escape from the blood vessels and infiltrate into the brain. The first layer is broken by their interaction with cerebral endothelial cells, which are damaged by tumor cells via activation of several signaling pathways. The small GTPase (Rho / ROCK) pathway, the PI3K / Akt pathway, and the TGF-β pathway are mainly considered to be involved in this interaction (83). A metastatic cancer cell that dislodges from the blood vessels due to collapse of the luminal structure, however, would probably be targeted by the exclusion network of the CNS; via promotion of plasmin generation from neuron-derived plasminogen by astrocyte, that cleaves Fas ligand (FasL) to kill cancer cell, and also by the immune cells immediately attacking a cancer cell if it exists as an independent cell (84). To escape this attack, a cancer cell often begins to interact with astrocytes in the brain. Integrins and GJs are attracting attention as molecules that mediate these heterogeneous cell-cell interactions. Details will be described later in section *2.6*.

In addition to its potential role in tumor invasion, Cx43 has been implicated in regulating cell death. One report indicates that Cx43 increases glioma cell resistance to apoptosis by a channel-dependent mechanism (85). We also demonstrate that Cx43 increases the resistance of human glioma cells to temozolomide treatment by modulating the mitochondrial apoptosis pathway (86). In addition, Cx43 interacts with Bax in the vicinity of the cell membrane, and by promoting its mitochondrial transition, it regulates the apoptotic signal of the cell and enhances sunitinib susceptibility (12). We will next review examples distinguishing whether the GJs mediate effects via homocellular-type or heterocellular-type of interactions in the multiple steps of cancer invasion. (Figure 1 and 2)

2.5. Homocellular Junctions

2.5.1. Glioma Channels in Invasion

We investigate the role of homocellular GJIC in glioma migration using a 3D spheroid migration model that mimics the in *vivo* architecture of tumor cells to quantify migration changes (87) and found that down-regulation of Cx43 expression in the U118 human glioma cell line increased migration by reducing cell-ECM adhesion, and changed the migration pattern from collective to single cell (87). More importantly, we are able to conclude that GJIC has a more prominent role in mediating migration in a 3D model. The ability of a C-terminal truncated Cx43 mutant (TrCx43) to produce migration levels similar to control cells expressing wild-type (WT) Cx43 suggests that the C-terminal is not mediating migration in this system (87). In contrast, we showed that blocking the channel function with a specific mutant (Cx43T154A) and a chemical channel blocker (carbenoxolone; CBX) increases migration suggesting that a significant reduction in GJIC is required to increase U118 glioma migration and/or invasion (Fig.1 pathway ②).

2.5.2. Homocellular Astrocyte Channels in Glioma Invasion

Upregulation of Cx43 has been detected in astrocytomas and peri-tumor parenchyma (88-90). Using an intracranial mouse model by which we implanted mCherry-labeled GL261 cells into the striatum of syngeneic C57B/6 mice with an intact immune system (91, 92), we similarly observed increased Cx43 immunoreactivity in the brain parenchyma within 100 μm from the edge of the tumor mass (66). Interestingly, Cx43 co-localizes with podoplanin (PDPN), a glycoprotein implicated to have a role in inflammation (93), in tumor-associated astrocytes (66). Our findings in an intracranial

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glioma mouse model demonstrate that the elimination of Cx43 in host astrocytes surrounding gliomas reduces the invasion of these tumor cells into the surrounding brain parenchyma (22). Despite robust intercellular communication between astrocytes was observed (Fig.1 pathway5), no significant increase in HC activity in the peri-tumor region (22), which is in contrast to what is expected from what we know about HC and inflammation (see previous section). In addition, our results with the T154A channel dead Cx43 mutant demonstrate that Cx43-mediated intercellular communication between glioma cells and astrocytes is not essential for astrocytic Cx43 to mediate its pro-invasive effect *in vivo*. Taken together, our results reveal an unexplored role of glial Cx43 paracrine signaling that can provide a permissive niche for glioma invasion (Fig.1 pathway4).

2.6. Heterocellular Junctions

2.6.1. Heterocellular GJIC in Glioma Invasion

Investigations on the specific roles of Cx43 in glioma invasion have been hindered by the fact that alteration in tumoral Cx43 levels not only disrupt intra-tumoral homocellular communication, but also perturb the formation of heterocellular channels between glioma cells and astrocytes. In addition, the opposing effects of homocellular and heterocellular GJIC on glioma migration (67) further complicates the role of tumoral Cx43 in glioma progression. As mentioned previously, our results (72) so far suggest that glioma-glioma GJs attenuate migration while glioma-astrocyte and astrocyte-astrocyte GJs promote glioma invasion. We speculate that the pro-invasive effects may arise from transfer of oncogenic signaling molecules from glioma cells to adjacent astrocytes via Cx43 (Fig.1 pathway 1), followed by spread of these signals (and/or their downstream effectors) among astrocytes through astrocyte-astrocyte GJs (Fig.1 pathway 5).

The direct passage of small ions and metabolites such as Ca^{2+} (33), ATP (2, 4, 34), glutamate (94), glucose (95), and peptides (96) through Cx43 channels are well known. However, increasing evidence has demonstrated that Cx43 is permeable to oligonucleotides as long as 24 nucleotides in length (97). Subsequently, siRNA and microRNA (miRNA) have been shown to exert functional effects on neighboring cells via GJs by using well established chemical inhibitors and reporter proteins such as GFP and luciferase (98-100). Indeed, we are able to demonstrate that glioma-astrocyte GJs are permeable to miRNAs, which are small, non-coding RNA molecules that often regulate several protein targets and signaling pathways, using cel-miR-67 as a "tracer" (72). Specifically, we identified a miR-5096 that enhance the invasiveness of glioma cells in a Cx43-dependent manner, by using a combination of techniques that include the use of GJ inhibitors and an anti-miR™ that 'neutralizes' the action of miR-5096 (72). The increase of miR-5096 is not due to endogenous upregulation by the astrocytes as we were unable to detect the corresponding much larger, GJ-impermeable primary miRNA that is the precursor of mature miR-5096 in the astrocytes (72). Our findings add to recent studies that demonstrate that cancer cells 'reprogram' normal stromal cells by miRNAs (101, 102). Astrocytes promote brain metastasis by silencing PTEN (phosphatase and tension homology), a protein well known for its tumour suppressive property, via miR-19a using exosomes as a delivery vehicle (103). On the other hand, it is also known that miR-5096 originating from glioma interacts with human microvascular cerebral endothelial cells (HMEC) through GJ and promotes angiogenesis (104) (Fig.1 pathway 3). Therefore, the microenvironment has a direct influence in the gene expression of tumour cells and glioma cells that directly influence astrocytes and HMEC to facilitate invasion.

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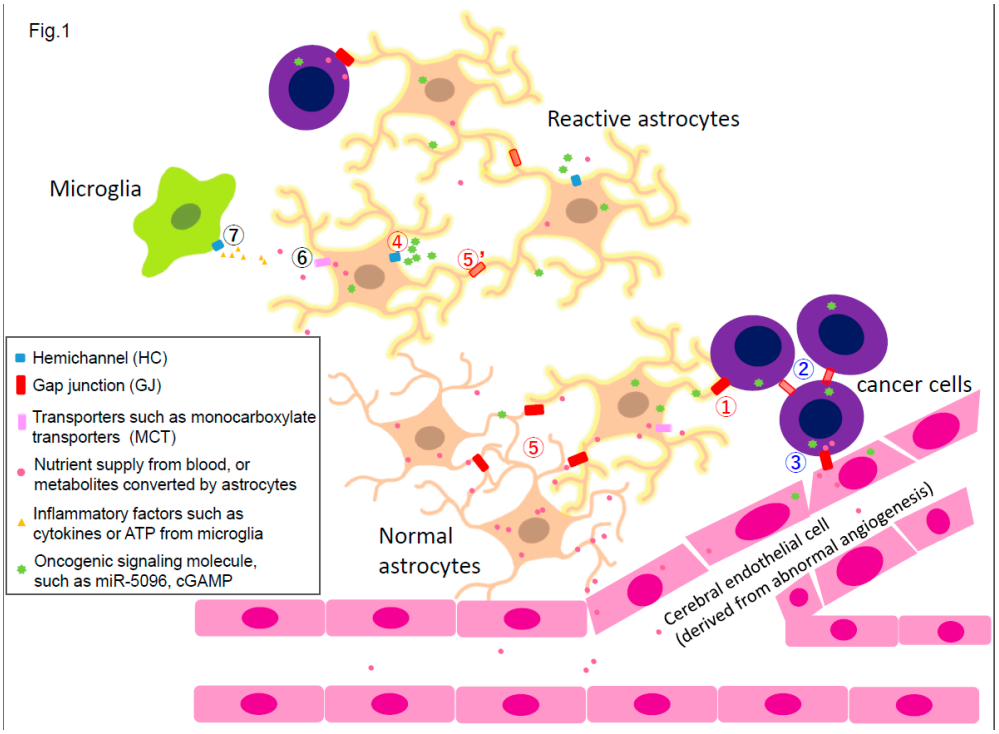

Figure 1. The microenvironment of glioma or metastatic cancers in central nerve system (CNS). Reactive astrocytes have increased Cx43 and GFAP levels, while PTEN, a well-known tumor suppressive protein, is decreased. Cerebral endothelial cells (CECs) also participated here as angiogenesis inducing factor and supply nutrients. Microglia increases glucose uptake by astrocytes. Numbers indicate GJ or HC pathways to maintain this environment. Red colored pathways (○1, ○4, ○5 and ○5′) are common between glioma and metastatic cancers. Blue colored pathways (○2 and ○3) are derived from previous reports on glioma (84,101). Black colored pathways (○6 and ○7) denotes those of metastatic cancers (3). ○1GJ between a cancer cell and an astrocyte; it promotes tumor invasion by transferring oncogenic signaling molecules from a cancer cell to an adjacent astrocyte. ○2GJ between glioma cells; down-regulation of GJ promotes invasion of glioma. ○3GJ between glioma and CEC; it promotes angiogenesis. ○4HC activity of reactive astrocyte; it contributes to spreading of oncogenic signaling molecule in the brain. ○5GJ between normal astrocytes; it supports nutrients supply from CECs to CNS cells. ○5GJ between astrocytes located distal from the tumor; it is mainly down-regulated when HC is upregulated. ○6Transporters expressed in astrocytes located distal from the tumor; they are believed to be upregulated to uptake enough metabolites. ○7HC activity in microglia; it may support increase of glucose uptake in astrocytes, while it decreases spread of glucose between astrocytes.

2.6.2. Heterocellular Gjs or Hcs: Extravascular Liberation of Metastatic Cancer - Neurovascular Unit Including Cerebral Endothelial Cells (Cecs), Pericytes, Glial Cells, and Neurons

For cancer cells to penetrate the CNS region, it is necessary to break through the blood brain barrier (BBB). BBB consists of CECs, pericytes, and glia (Fig.2). One factor that is essential for cancer metastasis is the condition of blood flow. For metastatic cells to undergo extravascular liberation, cells need to remain on the wall in the vascular lumen (Fig.2 pathway ③), therefore the infiltration process will be physically difficult to occur if the blood flow is too rapid. Substantial evidence suggests that the neurovascular unit facilitates cancer metastasis. High levels of Cx expression are observed at the endfeet of astrocytes that enclose the blood vessels (105, 106). An increase in $[Ca^{2+}]_i$ in astrocytes is observed during vasodilation or contraction (105, 107, 108) (Fig.2 pathway ②). The morphological changes of endothelial cells, such as the decision to contract or expand, depends on the nature of the signal that changes extracellular Ca^{2+} concentration. It is an interesting possibility that Cx43 present in the endfeet will affect the perivascular velocity of propagation of this $[Ca^{2+}]_i$ wave to adjacent astrocytes. It has been suggested that the expression of Cx channels increase the

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number of endfeet in contact with the CEC and is involved in regulating blood flow (105, 109, 110). It is also suggested that HCs are involved in $[Ca^{2+}]_i$ waves, possibly via ATP release regulating blood flow. Pericytes enclosing CECs have thick basement membrane, but its basement between astrocytes are thin and chemical exchange tends to occur. Furthermore, Cx43 HCs are present on the surface of pericytes and have been demonstrated to play an important role in the pericyte-mediated vascular network communication (111) (Fig.2 pathway ②). Thus, even at the capillary level, the blood flow rate is precisely controlled, and this regulation mechanism may be utilized by cancer cells when they infiltrate outside the blood vessel.

Immediately after cancer cells break through the BBB, metastatic cancer cells remain around the blood vessels. This perivascular region is not limited to cancer cells but also is a niche preferred by dormant cells such as neural and glial stem cells (112). An important progenitor cell proliferation pathway mediated by vascular endothelial growth factor (VEGF) occurs at this niche position, which is a favorable environment for metastatic spread of certain cancer cells (113). Therefore, the metastatic cancer cells tend to remain attached to the surface of the outer wall of the blood vessel (Fig.2 pathway ③). The GJ formed here supports this observation. Regarding the intracerebral infiltration of breast cancer and melanoma cells, GJs consisting of Cx26 and Cx43 have been demonstrated between CECs and invasive cancer cells, and CBX, a GJ inhibitor, was able to decrease the size or number of microcancer colonies in the brain (114). In addition, there are also microglia around the cerebral vascular unit. It is reported that microglia secrete cytokines and exosomes in the primary tumor of the brain (115, 116), which may contribute to proliferation in the brain environment of cancer cells located around blood vessels (Fig.2 pathway ④). It also activates PI3K / Akt signaling and IGF signaling, which in turn promotes angiogenesis in CECs (117). Further studies are needed to determine to what extent such functions of microglia support extravasation of metastatic cancer in the brain.

Fig.2

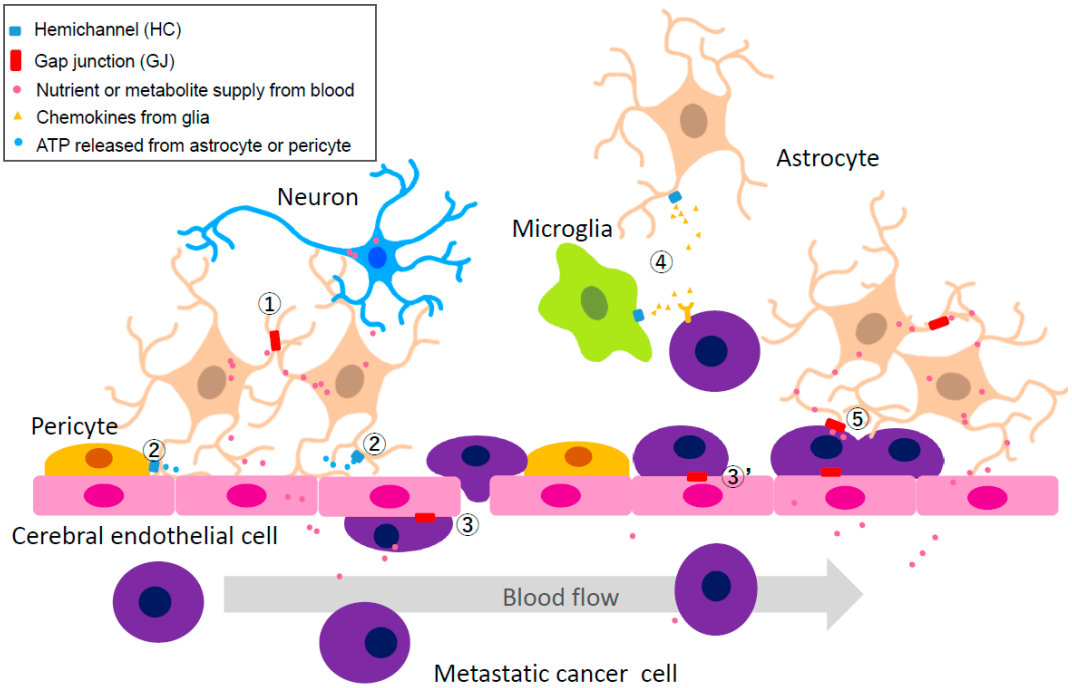

Figure 2. The peri-vascular niche of the tumor microenvironment highlighting the extravasation of metastatic cells. The neurovascular unit includes cerebral endothelial cells (CECs), pericytes, glial cells, and neurons. Metastatic cancer cells utilize the nutrient supply from CECs to CNS cells mediated by the astrocytes. ①GJ between normal astrocytes; it supports nutrients from CECs to CNS neurons. ②HC activity of astrocytes and pericytes; it adjusts $[Ca^{2+}]_i$ concentration in nearby CECs via release of ATP which results in change of blood flow rate. ③GJ between CEC and metastatic cancer

cell inside the capillary; it supports extravascular liberation of cancer cell from blood vessels. ③GJ between CEC and metastatic cancer cell located in a pericyte-like location; it protects cancer cells from immune attack by CNS astrocyte and microglia. ④HC activity of microglia or astrocyte; it supports cancer growth by release of chemokines, although the actual mechanisms by which glial cells promote immune attack or support cancer remains unknown. ⑤GJ between metastatic cancer cell located in a pericyte like-location and an astrocyte; it contributes to cancer growth as the first step in brain metastasis.

2.6.3. Heterocellular Gjs: Possibility of Utilizing Metabolic Coupling between Astrocytes and Neurons

It is reported that there exists approximately 86 billion neurons and 85 billion glia (sum of astrocyte, microglia, and other glia) in the whole human brain (118). The distribution ratio differ quite differently in each brain region: for instance, 1:3.7 in the cerebral cortex, and 1:11 in the brain stem. In particular, astrocytes residing around neurons play a key role for the maintenance of neuronal excitability: recycling neurotransmitters released into the synaptic cleft, buffering pH or K^+, and supplying neurons with antioxidants and metabolites (119, 120). GJs and HCs are intimately related to these astrocytic functions (Fig.2 pathway ①).

Recycling of glutamate, the primary excitatory neurotransmitter, is one of the critical roles for astrocytes since overstimulation of glutamic receptors is highly toxic to neurons. To prevent this excitotoxicity, the rapid uptake of glutamate is performed via glutamate transporters, like glutamate transporter 1 (GLT-1) and glutamate aspartate transporter (GLAST) expressed in astrocytes. It is shown that glutamate is able to directly pass through GJs, which is inhibited by the treatment of CBX, and the inhibition of GJs sensitizes neurons to glutamate toxicity (121, 122). These results suggest that the passage of glutamate or its metabolites through GJs may spread a signal among astrocytes so as to have them support the survival of neurons (Fig.2 pathway ①). In addition, glutamate uptake is accompanied by the co-transportation of Na^+, which generates a metabolic wave and evokes energy demand (123). The propagation of this metabolic wave is mediated by ions, and glycolytic metabolites like glucose and lactate also seem to be involved in the promotion of metabolism. Rouach *et al* revealed that a fluorescent glucose derivative 2-[N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino]-2-Deoxyglucose (2-NBDG) can pass through GJ between astrocytes, and the addition of glucose or lactate stimulates the neuronal activity in the mouse brain, which is abrogated in Cx43-knock out mice (124). This work suggests that the spread of glycolytic metabolites via astrocytic GJs also contributes to a signal transmission among astrocytes and positively affects neuronal activity. Monocarboxylate transporters (MCTs) are the only known lactate transport carriers, however, a recent study shows that HCs are related to the extracellular release of lactate and this phenomenon enhances synaptic transmission (125). Considering these reports, astrocytic GJs and HCs cooperate with each other and contribute to the astrocytic functions to support surrounding neurons. It has also been reported that cytokines secreted from microglia under inflammatory conditions enhance glucose uptake by astrocytes through HCs while reducing glucose diffusion through GJs between astrocytes (3) (Fig.1 pathway ④⑤⑥⑦), thus, the change of activity of HCs and GJs may be involved with the appearance of reactive astrocyte in inflammatory conditions. However, it is not known whether special metabolic networks may be developed in the cancerous lesions.

It is expected that the above astrocyte-neuron metabolic network occurs between astrocytes and invasive cancer cells (Fig.2 pathway ⑤). Cancer cells from the primary tumor move to the various organs via blood flow. As mentioned in the previous section, metastatic cancer cells which invaded into the BBB will probably contact astrocytes at first. Most of these cells die due to the action of astrocytes in the brain stroma. Astrocytes secretes plasminogen activator (PA) to generate plasmin from neuron-derived plasminogen, which promotes release of membrane-bound astrocytic FasL (84). It would be inducing a paracrine death signal in cancer cells. Additionally, another target of active plasmin is the L1CAM, adhesion molecule, thereby blocking interaction between cancer cells with the capillaries (84). On the other hand, cancer cells that survived the extravasation of blood vessels may utilize the heterocellular metabolic communication to adapt themselves to a new environment.

In our preliminary study, significant changes in intracellular metabolome were confirmed in both astrocytes co-cultured with culture supernatant of cancer cells and cancer cells co-cultured with astrocyte culture supernatant, respectively, compared with single cultures. Interestingly, the metabolome did not change if a main metabolite of the pathway is artificially added to the culture system of cancer cells or vice versa. It suggests that induction of metabolome change in each cell needs to continuous and bidirectional intercellular communication between astrocyte and a cancer cell, therefore GJs would be attractive target to realize this phenomenon.

2.6.4. . Heterocellular Gjs: Pro-Survival Functions of Gjs between Metastasized Cancer Cells and Astrocytes

Although astrocytes have important role to kick out cancer cells by using PA or FasL as mentioned above, they also help tumor survival and proliferation at the region; for example, it is observed that brain metastatic melanoma cells are surrounded by reactive astrocytes with morphological changes and highly expressed GFAP in the brain tissue of either human patients or cancer mouse models (126) (Fig.1 pathway ①). Moreover, direct contact with astrocytes confers the drug resistance on melanoma cells, which is inhibited by the treatment of CBX or Cx43-specific siRNA for astrocytes. These data suggest that a metastasized cancer cell and a reactive astrocyte are directly coupled via GJs, altering the intracellular survival signals (127), and promoting the survival of cancer cells.

One of the well-known second messengers passing through GJs is Ca^{2+} (33), and apoptosis is induced by the elevation of a $[Ca^{2+}]i$ (128). The previous report shows that astrocytic GJ is involved in the suppression of the rapid increase of $[Ca^{2+}]_i$ in melanoma cells induced by the treatment of anti-cancer agents, indicating that GJs contribute to sequestering the toxic signal transmitter (126). Another mechanism by which astrocytic GJs promote brain metastasis was elucidated by Chen *et al.* who revealed that cyclic dinucleotide cyclic GMP-AMP (cGAMP) is transmitted via GJs from brain metastatic breast and lung cancer cells to astrocytes, which promotes cancer cell growth (129) (Fig.1 pathway ①).

3. Conclusion

As we have seen above, GJ or HC composed by Cx43 are involved in many steps in the progression of brain cancer. The evidence is especially strong in metastatic cancers, in which GJs formed between CECs and a metastatic cancer cells is critical in the process of invasion from the outside of the brain through the blood vessel into the brain. Once in the brain tissue, the cancer cells have the opportunities to further integrate by communicating with astrocytes and microglia. These subsequent steps are predicted to be common in primary gliomas and brain metastasis. GJs formed between heterologous cells becomes a step of planting cancer seeds in allowing for its expansion in the brain environment. At this time, the substances communicated between heterologous cells include intracellular secondary messengers, microRNA and cGAMP. Moreover, there is a possibility that a cancer cell receives the gift of a metabolite which is originally used to benefit the neurons. Alternatively, toxins such as glutamate or an anti-cancer agent (either an effective drug metabolite; ganciclovir, or stress factor; Ca^{2+}) can flow from cancer cells to astrocytes, which helps to reduce toxicity in cancer cells. The prognosis is expected to depend on the critical role played by astrocytes, which make up the majority of glia constituting the brain parenchyma. Several reports suggest that the HC activity of the astrocyte is more suited for 'long range' signaling, instead of directly by GJs. Traditionally, GJs were often thought to function mainly in an anti-cancer context. Here, we discuss that the specific role of GJs, whether it is cancer suppressive or promoting, depends on the stages of cancer, whether they are undergoing extravasation or proliferation, and the identity of the cell types that make contact with the cancer cells. There has been a report that BBB-permeable GJ inhibitors can suppress brain metastasis in a mouse model (129); therefore, targeting the direct interaction between metastasized cancer cells and astrocytes may be a prominent therapeutic treatment for brain metastasis. Future identification of pathological targets and technological innovation that enhances the selectivity for targeting (selection of cells, choice of GJ or HC) will improve clinical applicability.

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