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Monocyte Chemoattractant Protein-1 and Blood-Brain Barrier

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Abstract

Blood-Brain Barrier (BBB) is a dynamic structure that maintains the homeostasis of the brain and thus proper neurological functions. BBB compromise has been found in many pathological conditions, including neuroinflammation. Monocyte chemoattractant protein-1 (MCP1), a chemokine that is transiently and significantly up-regulated during inflammation, is able to disrupt the integrity of BBB and modulate the progression of various diseases, including excitotoxic injury and hemorrhage. In this review, we first introduce the biochemistry and biology of MCP1, and then summarize the effects of MCP1 on BBB integrity as well as individual BBB components.

Monocyte chemoattractant protein-1 (MCP1, also known as CCL2) is a pro-inflammatory mediator, whose up-regulation is found in many central nervous system (CNS) disorders with blood brain barrier (BBB) breakdown. Accumulating evidence suggests that MCP1 is able to compromise the integrity of BBB and modulate the progression of various diseases. Although several studies exploring the effects of MCP1 on BBB permeability regulation, the exact mechanism, especially the effects of MCP1 on pericytes, astrocytes, neurons, and basement membrane (BM), remain elusive. Here we review the biochemical and biological functions of MCP1 with a focus on its role in BBB regulation. Understanding the molecular mechanisms underlying MCP1-induced BBB disruption not only broadens our knowledge on chemokines and BBB, but also promotes the development of novel therapeutic reagents for many CNS diseases.

1. CHEMOKINES

Chemokines are a superfamily of structurally related small basic proteins with strong chemotactic activity. They function to induce cell-specific migration and activation of cells, especially immune cells, in response to insults (Glabinski et al., 1996; Hulkower et al., 1993; Lahrtz et al., 1998; Miller and Meucci, 1999). After the first chemokine was identified in 1977 (Wu et al., 1977), many chemokines and chemokine receptors have been identified (Ruffini et al., 2007). Based on the number and position of conserved cysteine on their primary sequences, chemokines are divided into 4 sub-types: C, CC, CXC, and CXXXC (Murphy, 1994; Rollins, 1997; Yoshie et al., 1997). The major functions of these chemokines include recruiting leukocytes during inflammatory conditions, maintaining ligand homeostasis between blood and tissue, and regulating developmental processes/ disease progression, such as BBB permeability and myelination (Campbell and Butcher, 2000; Cardona et al., 2008; Tran and Miller, 2003). These biological functions are mediated through G-protein-coupled receptors (Ransohoff, 2002). Studies on chemokines and their receptors reveal promiscuity: one receptor may have more than one ligands and one ligand

may have more than one receptors, which adds complexity to the studies of chemokines and chemokine receptors.

2. MCP1

MCP1, one of the most highly and transiently expressed chemokines during inflammation, is a member of CC sub-type chemokines. In the brain, MCP1 is expressed by most cell types, including neurons, astrocytes, microglia and BMEC (Andjelkovic et al., 1999a; Andjelkovic and Pachter, 2000; Andjelkovic et al., 1999b; Banisadr et al., 2005; Boddeke et al., 1999; Dicou et al., 2004; Horuk et al., 1997; Kalehua et al., 2004; Mahad and Ransohoff, 2003; Mahajan et al., 2005; Meeuwsen et al., 2005; Mennicken et al., 1999; Ransohoff et al., 1993; Storer et al., 2005; Wittendorp et al., 2004; Zeng et al., 2005). MCP1 is synthesized with a signal peptide in its N-terminus, which is removed during secretion (Yoshimura et al., 1989). The secreted MCP1 binds to soluble glycosaminoglycans (GAG) and GAG immobilized on cell surface and the extracellular matrix (Hoogewerf et al., 1997; Kuschert et al., 1999; Middleton et al., 1997; Middleton et al., 2002; Rot, 1992; Rot, 1993; Wagner et al., 1998). This interaction is predicted to induce dimerization/oligomerization of MCP1, increase its local concentration, and promote formation of chemokine gradients (Hoogewerf et al., 1997; Lau et al., 2004; Wang et al., 2005).

Although the N-terminus of MCP1 is highly homologous among different species, the C-terminus is not. Human MCP1 has 76 amino acids, whereas mouse MCP1 has a C-terminal extension with about 50 amino acids. It has been shown that the C-terminal extension of mouse MCP1 is heavily O-glycosylated (Ernst et al., 1994). What is the function of the C-terminal extension of mouse MCP1? We have previously been able to show that plasmin cleaves mouse MCP1 and removes its C-terminal extension (see Microglia section for details). Using recombinant wild-type and modified mouse MCP1, we further show that wild-type mouse MCP1 is able to dimerize and the mutant without C-terminus fails to dimerize (Yao and Tsirka, 2010). Additionally, the C-terminus alone interacts with neither wild-type nor mutant mouse MCP1, suggesting that the highly glycosylated C-terminal extension of mouse MCP1 is necessary but not sufficient for dimerization. Surprisingly, human MCP1, which is highly homologous to the N-terminus of mouse MCP1, forms dimer in physiological concentration and the residues crucial for the dimerization have been identified as amino acids 6–16 (Zhang and Rollins, 1995). Echoed with this report, two mutant forms of human MCP1, P8A and Y13A, have been reported to be unable to dimerize (Paavola et al., 1998).

Being able to form a dimer does not necessarily mean it functions as a dimer. The next question is whether MCP1 functions as a monomer or dimer. Zhang and Rollins showed that chemical-crosslinked human MCP1 dimer was functional in attracting monocytes *in vitro* (Zhang and Rollins, 1995). Furthermore, a mutant form of MCP1, 7ND, which lacks residues 2–8, has been shown to inhibit the function of wild-type MCP1, but not crosslinked MCP1 (Zhang and Rollins, 1995), suggesting that 7ND is a dominant-negative mutant and MCP1 functions as a dimer. P8A mutant MCP1, on the other hand, has wild-type binding affinity for CCR2 and induces calcium influx and chemotaxis at the same level as wild-type MCP1 (Paavola et al., 1998), suggesting MCP1 works as a monomer. Additionally, 7ND MCP1 has also been shown to function as a competitive inhibitor of monomeric MCP1 (Paavola et al., 1998). Consistent with the monomer theory, we have previously been able to demonstrate that compared to full length mouse MCP1, the mutant variant without C-terminus, which can not form a dimer, more efficiently activates Rac1 and promotes the formation of lamellipodia (Yao and Tsirka, 2010).

MCP1 exerts its biological functions by binding to its high affinity receptor, CCR2, which is mainly expressed by microglia, astrocytes and BMEC in the brain (Banisadr et al., 2002; Ge et al., 2008). Although MCP1 has only one high affinity receptor, CCR2 has four more ligands (CCL7, CCL8, CCL12, and CCL13) besides MCP1 (Gong et al., 1997; Gouwy et al., 2004; Wain et al., 2002). In rodents, only one CCR2 isoform is found, whereas two alternatively spliced CCR2 isoforms with different C-terminus (Charo et al., 1994) are found in human, denoted CCR2A and CCR2B. CCR2B is mainly expressed on monocytes and activated NK cells, whereas mononuclear cells and vascular smooth muscle cells predominately express CCR2A (Bartoli et al., 2001).

3. MCP1 AND BBB

The BBB is the largest CNS-barrier, which sustains brain homeostasis and thus proper neurological functions. At the BBB, specialized endothelial cells (brain microvascular endothelial cells, BMECs), astrocytes, pericytes, basement membrane (BM), neurons, and microglia can be found (Guillemin and Brew, 2004). The BMECs connect to each other via a complex network of tight junctions, which create the primary barrier and prevent paracellular transport across endothelial cells. These cells deposit a layer of BM (endothelial BM), in which pericytes are embedded. Astrocytes, which wrap BMECs and pericytes with their endfeet, deposit another layer of BM (parenchymal BM). These two layers of BM are not distinguishable except at the post-capillary venules, where a cerebrospinal fluid-drained perivascular space separates them. Neurons and microglia, which exist in the parenchyma, have direct contact with astrocytes and BMECs. The above mentioned cells together with both endothelial and parenchymal BM are necessary for an intact barrier of the BBB (Engelhardt and Ransohoff, 2012). Perturbation of BBB has been found in many neurological conditions, including trauma (Barzo et al., 1997; Shlosberg et al., 2010), brain tumors (Jain et al., 2007; Papadopoulos et al., 2001), stroke (Belayev et al., 1996; Benchenane et al., 2004; Moskowitz et al., 2010), and neurodegenerative diseases (Bell et al., 2010; Iadecola, 2010; Kortekaas et al., 2005; Selkoe, 2004; Zlokovic, 2005), and BBB breakdown is one of the hallmarks that accompany the progression of these diseases. Recently, BBB compromise has been found to play a causative role in the onset of Alzheimer's disease (Bell et al., 2010) and amyotrophic lateral sclerosis (Garbuzova-Davis et al., 2011). A large number of molecules have been reported to affect the permeability of BBB, such as monocyte chemoattractant protein-1 (MCP1), TNF- α , IL-1 β , IL-10, and IFN- γ (Blamire et al., 2000; Oshima et al., 2001; Stamatovic et al., 2006; Stamatovic et al., 2003; Stamatovic et al., 2005; Wojciak-Stothard et al., 1998; Yang et al., 1999; Yao and Tsirka, 2011a; Yao and Tsirka, 2011b). Here we focus on the effect MCP1 on BBB integrity.

It has been shown that injection of recombinant mouse MCP1 into the brain disrupts BBB integrity (Dimitrijevic et al., 2006; Stamatovic et al., 2006; Stamatovic et al., 2003; Stamatovic et al., 2005; Yao and Tsirka, 2011b). We have previously been able to further demonstrate that plasmin-mediated truncation of MCP1 is indispensable for MCP1-induced BBB compromise (Yao and Tsirka, 2011b). Consistent with our report, tPA, which converts inactive plasminogen to active plasmin, has been found to promote BBB disruption and subsequent peripheral blood mononuclear cell (PBMC) infiltration (Reijerkerk et al., 2008). Additionally, BBB compromise and PBMC infiltration have also been found in mice deficient for plasminogen activator inhibitor-1 (Kataoka et al., 2000), suggesting that the effect of MCP1 on BBB is dependent on plasmin activity. MCP1, however, failed to compromise BBB integrity in CCR2^{-/-} mice (Stamatovic et al., 2005), suggesting that the effect of MCP1 on BBB also depends on CCR2. The next question then becomes how exactly MCP1-CCR2 axis affects the integrity of BBB. The roles of MCP1-CCR2 on individual BBB components are summarized below.

3.1. BMECs

The capillary endothelium in the brain is 50–100 times tighter than that in the periphery (Abbott, 2002). Compared with peripheral endothelial cells, BMECs are characterized by the presence of more mitochondria, less pinocytotic activity, lack of fenestrations, and the presence of tight junctions (Fenstermacher et al., 1988; Kniesel and Wolburg, 2000; Oldendorf et al., 1977; Sedlakova et al., 1999). BMECs connect to one another forming an impermeable monolayer. In the interendothelial space specific structures, including adherens junctions and tight junctions, are present (Kniesel and Wolburg, 2000; Schulze and Firth, 1993; Vorbrodt and Dobrogowska, 2003; Wolburg and Lippoldt, 2002). Although both adherens and tight junctions act to limit paracellular permeability of endothelial cells (Bazzoni and Dejana, 2004), tight junctions are the primary structure that maintains the impermeability of BBB (Romero et al., 2003). In the tight junctions, many tight junction proteins are expressed. There are two types of tight junction proteins: transmembrane ones, such as occludin and claudin-1, 5, 11, and cytoplasmic accessory proteins, such as zonula occluden-1, 2, 3 (ZO-1, 2, 3) and cingulin (Citi and Cordenonsi, 1998; Huber et al., 2001). The transmembrane proteins, especially occludin, function to seal gaps between adjacent cells (Forster, 2008; Michel and Curry, 1999; Mitic and Anderson, 1998; Ogunrinade et al., 2002). Occludin, a 60–65kD transmembrane protein with its amino- and carboxyl-terminus in the cytoplasm, has been shown to maintain the integrity of BBB (Bolton et al., 1998; Brown and Davis, 2005; Huber et al., 2002; McCarthy et al., 1996). There is also evidence showing that the phosphorylation state of occludin regulates BBB integrity by affecting its membrane association (Andreeva et al., 2001; Clarke et al., 2000b; Hirase et al., 2001; Kale et al., 2003; Rao et al., 2002; Sakakibara et al., 1997; Wachtel et al., 1999). Cytoplasmic accessory proteins, on the other hand, link transmembrane proteins to cortical actin-based cytoskeleton (Forster, 2008; Michel and Curry, 1999; Mitic and Anderson, 1998; Ogunrinade et al., 2002). ZO-1, the first identified accessory protein (Stevenson et al., 1986) that links occludin to actin cytoskeleton (Fanning et al., 1998), plays a critical role in regulating BBB permeability. Dissociation of ZO-1 from the tight junctions has been shown to accompany the disruption of BBB (Abbruscato et al., 2002; Fischer et al., 2002; Mark and Davis, 2002; Yao and Tsirka, 2011a; Yao and Tsirka, 2011b). In addition, ZO-1 has been detected in the nucleus and shown to co-localize with transcription factors in some conditions (Balda and Matter, 2000; Gottardi et al., 1996; Hawkins et al., 2004; Riesen et al., 2002), suggesting its potential role as a signaling molecule.

BMECs as the major barrier of BBB have been under extensive investigations. Accumulating evidence shows that MCP1 compromises BBB integrity via redistribution of tight junction proteins from cell-cell border (probable via endocytosis) and reorganization of actin cytoskeleton in the BMEC (Dimitrijevic et al., 2006; Stamatovic et al., 2006; Stamatovic et al., 2003; Stamatovic et al., 2005). We have previously been able to verify these data and further show that these changes are dependent on plasmin activity (Yao and Tsirka, 2011b). Mechanistic studies reveal that phosphorylation of TJP regulates their functions and locations (Clarke et al., 2000a; Clarke et al., 2000b; Farshori and Kachar, 1999; Hirase et al., 2001; Ward et al., 2002). Stamatovic and colleagues further demonstrated that the binding of MCP1 to CCR2 activated PKC (specifically PKC α and PKC ζ) and Rho kinase, resulting in shift of tight junction proteins from cell border to intracellular compartments (Stamatovic et al., 2006; Stamatovic et al., 2003). Additionally, this phosphorylation event also promoted the interaction between tight junction proteins and actin cytoskeleton, resulting in a shift of tight junction proteins from Triton X-100 soluble fraction to Triton X-100 insoluble fraction (Stamatovic et al., 2006; Stamatovic et al., 2003; Stamatovic et al., 2005; Tsukamoto and Nigam, 1997; Tsukamoto and Nigam, 1999). These changes are not limited to MCP1, because growth factors (PDGF and VEGF) also induce phosphorylation and redistribution of tight junction proteins (Harhaj et al., 2002; Pedram et al., 2002), suggesting phosphorylation

of tight junction proteins may be a common mechanism to transport them to different cellular compartments. In addition to tight junction protein phosphorylation, the activated kinases, especially Rho Kinase, also phosphorylate myosin light chain phosphatase (MLCP) and inhibit its activity. The inhibition of MLCP results in enhanced phosphorylation of myosin light chain, leading to increased actin-myosin interaction and thus increased cortical force in the endothelial cells (Hicks et al., 2010; Stamatovic et al., 2003; Stephan and Brock, 1996; van Nieuw Amerongen et al., 2000). In addition, we have previously been able to demonstrate that binding of MCP1 to CCR2 on BMECs also promotes phosphorylation of Ezrin/Radixin/Moesin (ERM) proteins on conserved Threonine residues (Thr567 for ezrin, Thr564 for radixin, and Thr558 for moesin) (Yao and Tsirka, 2011b). ERM proteins are a family of highly conserved proteins that act as a linker between plasma membrane and actin cytoskeleton. Unphosphorylated ERM proteins form intramolecular interaction between N- and C-terminus. When phosphorylated on the conserved Threonine residues, they form intermolecular interaction (N-terminus binding to membrane proteins and C-terminus binding to actin cytoskeleton) (Bretscher et al., 1995; Louvet-Vallee, 2000; Pearson et al., 2000). Our data showed that MCP1 treatment led to phosphorylation of ERM proteins and promoted interaction between ZO-1 and phosphorylated ERM proteins (Yao and Tsirka, 2011b), indicating an important role of ERM proteins in translocation of ZO-1 upon MCP1 treatment. Occludin, however, did not interact with ERM proteins even upon MCP1 treatment (Yao and Tsirka, 2011b), indicating different ways of regulation. Taken together, we proposed a mechanism responsible for MCP1 induced BMEC and thus BBB changes as shown in Figure 1. The binding of MCP1 to CCR2 activates unknown kinase(s), which phosphorylates ERM proteins. The phosphorylated ERM proteins then bind to ZO-1 and actin cytoskeleton. In addition, Rho associated kinase, which is activated by MCP1, phosphorylates MLCP, resulting in decreased phosphatase activity. The imbalance of MLCP and MLC kinase activity results in over-phosphorylation of MLC, and thus increased and prolonged actin-myosin contraction. This contraction in turn pulls ZO-1 away from tight junction complex, leading to disruption of BBB integrity. In the BMEC-astrocyte co-culture system, Stamatovic and colleagues further demonstrated that lack of expression of CCR2 in BMEC was sufficient to prevent the leakage of BBB upon MCP1 treatment (Stamatovic et al., 2005), suggesting that MCP1 induced BBB compromise is dependent on endothelial CCR2.

3.2. Astrocytes

The unique properties of BMECs (increased mitochondria number, few pinocytotic vesicles and presence of tight junctions (Fenstermacher et al., 1988; Kniessel and Wolburg, 2000; Oldendorf et al., 1977; Sedlakova et al., 1999)) lead to the question: is it due to intrinsic characteristics of BMECs or the microenvironment in the brain? Stewart and Wiley elegantly demonstrated that nonvascularized brain tissue grafted into coelomic cavity developed capillaries with BMEC properties, whereas somite tissue grafted into cerebral ventricles failed to do so (Stewart and Wiley, 1981), strongly suggesting that interaction between vascular tissue and CNS tissue contributes to the properties of BMECs. In the brain, astrocytic endfeet together with the parenchymal BM establish the glia limitans, which defines the parenchymal border in the CNS (Engelhardt and Ransohoff, 2012). Astrocytic endfeet cover more than 99% of the vascular surface (Kacem et al., 1998; Simard et al., 2003), suggesting that astrocytes may confer BMECs those unique properties and thus contribute to the impermeability of BBB. Janzer and Raff found that astrocytes induced tight junction in endothelial cells within the eyes (Janzer and Raff, 1987), indicating that astrocytes can enhance the impermeability of endothelial cells lining the capillaries in the eyes. In data consistent with these, the BMEC-astrocyte co-culture system showed a higher transendothelial electrical resistance (TEER) and less infiltration of tracers across the *in vitro* BBB than BMECs alone (Cohen-Kashi Malina et al., 2009; Neuhaus et al., 1991;

Siddharthan et al., 2007; Tao-Cheng et al., 1987; Yao and Tsirka, 2011b). Additionally, the temporary focal loss of astrocytes has been found to parallel the compromise of BBB integrity *in vivo* (Willis et al., 2004). Further experiments reveal that the role of astrocytes in BBB integrity is due to the release of soluble factors, including Ang1, TGF- β , GDNF, and FGF2 (Cardoso et al., 2010; Persidsky et al., 2006; Quaegebeur et al., 2011), as well as the direct contact with BMECs (Lai and Kuo, 2005). Recently, polarized distribution of intramembranous orthogonic arrays of particles (OAPs), which contain water channel aquaporin 4 (AQP4), the potassium channel Kir4.1, and a dystroglycan-dystrophin complex, has been reported in astrocytic endfeet (Wolburg et al., 2011). Given the functions of AQP4 and Kir4.1 (regulation of water and ion homeostasis at glial-endothelial interface, respectively), OAPs have been speculated to influence BBB permeability (Quaegebeur et al., 2011; Wolburg et al., 2011). Together, these data support that astrocytes contribute to the impermeability of BBB. It should be noted that the effect of astrocytes in BBB integrity only takes place in adulthood, because astrocyte development starts after birth (Daneman et al., 2010). There is also evidence, however, suggesting astrocytes may not contribute to the BBB integrity (Krum et al., 1997). This inconsistency may be due to different experimental conditions or methodology.

Since astrocytes are a main source of MCP1, transgenic mice over-expressing MCP1 in astrocytes have been developed and used to study the function of MCP1. In addition to enhanced nociceptive responses in these transgenic mice (Menetski et al., 2007), chronic expression of MCP1 in astrocytes induces BBB compromise *in vivo*, indicating a crucial role of astrocytic MCP1 in BBB regulation (Huang et al., 2005). In an *in vitro* system, it has been shown that MCP1 binding in human astrocytes is mediated by both CCR2 and D6 decoy chemokine receptor (Fouillet et al., 2012) and activation of CCR2 promotes the survival of astrocytes (Quinones et al., 2008). Additionally, caveolin-1 has been found to mediate the effects of MCP1 in astrocytes (Ge and Pachter, 2004). Knockout of CCR2 in astrocytes, however, failed to affect BBB integrity in the BMEC-astrocyte co-culture model (Stamatovic et al., 2005), suggesting that CCR2 in astrocytes is dispensable for MCP1 induced BBB breakdown. This discrepancy may be due to the over-simplified *in vitro* BBB models used. Future studies should focus on *in vivo* studies or use *in vitro* BBB models that replicate both the anatomical and physiological characteristics of the BBB.

3.3. BM

Two layers of BM are found at the BBB: endothelial BM and parenchymal BM (Engelhardt and Ransohoff, 2012; Owens et al., 2008). The two layers are morphologically indistinguishable except at the post-capillary venules, where a cerebrospinal fluid-drained perivascular space separates them (Engelhardt and Ransohoff, 2012). BM consists of a mixture of extracellular matrix (ECM) proteins, including collagens, laminins, heparin sulfate proteoglycans, fibronectin, vitronectin, nidogens, perlecan, and agrin (Agrawal et al., 2006; Baumann et al., 2009; Cardoso et al., 2010; Fukuda et al., 2004; Yurchenco et al., 2004). BM assembly mainly involves polymerization of laminins and collagens, which is cross-linked by nidogens (Yurchenco et al., 2004; Yurchenco and Patton, 2009). Although most ECM proteins are ubiquitously expressed at both endothelial and parenchymal BM, the former mainly expresses laminin $\alpha 4$ and $\alpha 5$ (Sixt et al., 2001) and the latter predominantly expresses laminin $\alpha 1$ and $\alpha 2$ (Owens et al., 2008; Sixt et al., 2001; van Horssen et al., 2005).

BM has been proposed to regulate BBB integrity directly as a physical barrier (Barber and Lieth, 1997), given its anatomical location: between BMECs and astrocytes. Loss of BM leads to breakdown of BBB (del Zoppo, 2009; Del Zoppo et al., 2006; Kwon et al., 2009; Lo and Rosenberg, 2009; Wang and Shuaib, 2007; Zhang et al., 2007). In addition, the BM also contributes to the integrity of BBB through individual ECM proteins, which not only anchor

different cells in place at BBB, but also regulate cellular processes (Agrawal et al., 2006; Cardoso et al., 2010; Owens et al., 2008; Sixt et al., 2001; Tilling et al., 2002). It has been shown that laminin, collagen type IV, and fibronectin increase TEER of brain capillary endothelial cells (Tilling et al., 1998). Dystroglycan, a major receptor for ECM proteins, is selectively cleaved at the parenchymal BM at site of leukocyte infiltration during experimental autoimmune encephalomyelitis (Agrawal et al., 2006). Additionally, laminin $\alpha 5$, an endothelial BM specific component, has been shown to serve as a barrier for leukocyte translocation (Sixt et al., 2001). These results suggest that ECM proteins and their receptors also contribute to the integrity of BBB.

Consistent with the detrimental role of MCP1 in BBB integrity, MCP1 has been found to promote ECM protein degradation induced by hydrogen peroxide (Kim et al., 2013). Contrary to this finding, there are reports showing that MCP1 directly contributes to the production and accumulation of ECM, especially collagen, in many disease models, including diabetic nephropathy (Park et al., 2008), systemic sclerosis (Arai et al., 2013), and peritoneal dialysis-related epithelial-mesenchymal transition (Lee et al., 2012). This discrepancy may be explained by different disease models and/or indirect effects of MCP1. For example, leukocytes recruited by MCP1 may secrete proteases that degrade ECM proteins (Greenlee et al., 2007; Stamenkovic, 2003). The exact role of MCP1 in BM needs further investigations.

3.4. Pericytes

Discovered more than 100 years ago (Rouget, 1873), pericytes are sandwiched in between the abluminal side of BMECs and luminal side of astrocytic endfeet (Sa-Pereira et al., 2012b). Specifically, pericytes are embedded in the endothelial BM in CNS capillaries (Armulik et al., 2011; Engelhardt and Ransohoff, 2012) and the degree of its coverage on endothelial cells varies depending on tissue type and species (Sa-Pereira et al., 2012b). It has been shown that the pericyte-to-endothelial ratio is 1:1 in retina, 1:3 in brain, and 1:100 in skeletal muscles, respectively (Dalkara et al., 2011; Sa-Pereira et al., 2012a). The average pericyte-to-endothelial ratio is lower in rat capillaries (1:5) and relatively higher in mouse and human capillaries (1:4 and 1:3–4, respectively) (Dore-Duffy, 2008; Dore-Duffy and Cleary, 2011). Although brain capillaries have high pericyte coverage, the precise percentage of capillary surface covered by pericytes varies significantly depending on different research groups, ranging from 22–99% (Dalkara et al., 2011; Engelhardt and Sorokin, 2009; Frank et al., 1987). This difference may be due to the different pericyte markers used. There are no specific markers for pericytes so far, although several markers, including α -smooth muscle actin (SMA) (Dore-Duffy, 2008; Fisher, 2009; Krueger and Bechmann, 2010; Nehls and Drenckhahn, 1993), PDGFR β (Lindahl et al., 1997; Winkler et al., 2010), Desmin (Nehls et al., 1992), CD13 (Dermietzel and Krause, 1991; Kunz et al., 1994), NG2 (Huang et al., 2010; Ozerdem et al., 2001; Ruitter et al., 1993), and RGS-5 (Bondjers et al., 2003; Cho et al., 2003), have been used to identify pericytes. It should be noted that (1) these markers are also expressed by other types of cells besides pericytes, such as smooth muscle cells, myofibroblasts, and neuronal progenitors (Armulik et al., 2011; Bandopadhyay et al., 2001; Nehls and Drenckhahn, 1991), and (2) the expression of these markers also depends on the differentiation stage of pericytes. It has been shown that TGF- β treated (further differentiated) pericytes express significantly more SMA, VEGF, MMP-2 and MMP-9 than bFGF treated (less differentiated) pericytes (Thanabalasundaram et al., 2011). Fate mapping experiments have shown that pericytes have several different developmental origins (Armulik et al., 2011). For example, ectoderm-derived neural crest gives rise to pericytes in the brain and thymus (Etchevers et al., 2001; Heglind et al., 2005; Korn et al., 2002), whereas pericytes in the lungs (Que et al., 2008), liver (Asahina et al., 2011), and gut (Wilm et al., 2005) are from mesothelium. In addition, there is also evidence

suggesting that some pericytes are derived from hematopoietic stem cells (Hess et al., 2004; Krueger and Bechmann, 2010).

Pericytes have been reported to exert different functions, including BBB regulation, vascular development, homeostasis maintenance, and serving as contractile and multipotent cells (Dore-Duffy, 2008; Dore-Duffy et al., 2006; Fisher, 2009). Here we only focus on its role in BBB regulation. It has been found that addition of pericytes to BMEC-astrocyte co-culture system significantly enhances TEER (Dente et al., 2001; Nakagawa et al., 2009), suggesting that pericytes contribute to the integrity of BBB. In addition, pericytes have been shown to up-regulate P-glycoprotein functional activity in endothelial cells and control tight junction permeability (Dohgu et al., 2005; Edelman et al., 2006; Peppiatt et al., 2006), suggesting that the interaction between endothelial cells and pericytes may play an important role in BBB integrity regulation. Using mice with defects in pericyte generation, researchers have demonstrated that pericyte coverage positively correlates with tight junction tightness (Lai and Kuo, 2005) and negatively correlates with the BBB permeability (Daneman et al., 2010). Consistently, a correlation between pericyte loss and BBB breakdown has been reported (Armulik et al., 2010; Bell et al., 2010). Further mechanistic studies suggest that the diminished expression of BBB-specific genes in endothelial cells and lack of polarity in astrocytic endfeet are responsible for pericyte loss induced BBB breakdown (Armulik et al., 2010). In pathological conditions, such as hypoxia or traumatic brain injury, which lead to the disruption of BBB, pericytes have been found to migrate away from the micro-vasculatures (Dore-Duffy et al., 2000; Gonul et al., 2002), but the relationship between migration of pericytes and compromise of BBB has not yet been studied.

Increased MCP1 levels have been reported in many disorders, including neurodegeneration, neuroinflammation, and kidney fibrosis (Chen et al., 2011; Sheehan et al., 2007). Additionally, MCP1 concentration has also been revealed to correlate with heavily oxidized LDL-induced pericyte injury (Zhang et al., 2008). However, due to the recent discovery of the critical role of pericytes in BBB regulation, only limited data are available to answer the question how MCP1 affects pericytes.

3.5. Microglia

Microglia, which account for 10–20% of glial cells, are brain resident immune cells. Their number in the brain (100–200 billion depending on the condition) is comparable to that of neurons. It had long been believed that microglia originated from myeloid progenitors in the bone marrow (Chan et al., 2007; Hailer et al., 1997; Ling, 1979; Ono et al., 1999; Wu et al., 2000) and these macrophage-like cells migrated into the brain during early development (before the formation of BBB). Recent lineage tracing study has shown that adult microglia derive from colony stimulating factor-1 receptor (CSF1R) positive primitive myeloid progenitors that arise before embryonic day 8 (Ginhoux et al., 2010). Consistently, fate-mapping experiments support that microglia mostly originate from *Myb*-independent, *FLT3*-independent, but *PU.1*-dependent myeloid progenitors that express CSF1R at embryonic day 8.5 (Buza-Vidas et al., 2011; Erblich et al., 2011; Fantin et al., 2011; Gomez Perdiguero et al., 2013; Qian et al., 2011; Schulz et al., 2012). Microglia have two states: a resting state with ramified morphology and an activated state with amoeboid morphology. Compared to the amoeboid morphology, the ramified structure has a smaller cell body surrounded by many long, thin, and highly dynamic processes. In the brain parenchyma, ramified microglia extend and retract their processes continually to sense changes in the surrounding microenvironment (Nimmerjahn et al., 2005). It is estimated that microglia can survey the entire brain in a few hours (Nimmerjahn et al., 2005). When there is an injury or disturbance of homeostasis in the CNS, microglia become activated. The activation involves change of morphology and gene expression. The activated microglia then migrate to the injury site and

proliferate locally. Additionally, these cells also secrete both pro- and anti-inflammatory cytokines, phagocytose cellular debris, process antigens and present them to T cells via MHC class I molecules (Abromson-Leeman et al., 1993; Aloisi, 2001; Giulian and Baker, 1986; Hanisch and Kettenmann, 2007; Kim and de Vellis, 2005; Nakajima and Kohsaka, 2004; Suzumura et al., 1987; Ulvestad et al., 1994). Whether microglia play a beneficial or detrimental role in CNS injury is highly controversial. There is evidence showing that microglia play neuroprotective roles by clearing cell debris and secreting factors promoting neurite growth and neuronal survival, such as neurotrophin-3 and brain-derived neurotrophic factor (BDNF) (Elkabes et al., 1996; Nakajima et al., 2002; Rabchevsky and Streit, 1997). On the contrary, microglia have also been shown to produce pro-inflammatory cytokines, including TNF- α and IL-1 β , which induce direct cytotoxicity by binding to their receptors (TNFRs and IL-1RI, respectively) and activating downstream cell death pathways (MacEwan, 2002). Given that microglia are found in the perivascular space, it is speculated that microglia may play an important role in regulating BBB integrity by interacting with other BBB components, including BMECs, astrocyte endfeet, and pericytes (Choi and Kim, 2008). There is evidence showing that microglial activation restores BBB integrity after the disruption of BBB (Willis, 2011). On the contrary, TNF- α released from activated microglia has been shown to impair BBB integrity (Nishioku et al., 2010), possibly through TNF- α induced direct cytotoxicity on BMECs. This discrepancy may be due to different injury models and different timing after injury. Therefore, further investigations are needed to clarify the role of microglia in BBB permeability.

As indicated by its name, MCP1 is a potent chemoattractant for monocytes and microglia. MCP1 induced chemotaxis of monocytes and microglia has been found in many CNS injuries, including ischemia, excitotoxicity and hemorrhage (Capoccia et al., 2008; Dimitrijevic et al., 2006; Frangogiannis et al., 2007; Hanisch and Kettenmann, 2007; Kim et al., 2008; Morimoto et al., 2008; Sheehan et al., 2007; Yan et al., 2007). The trafficking of microglia and leukocytes, however, is impaired in mice lacking CCR2, suggesting that MCP1 induced chemotaxis is dependent on CCR2 (Chen et al., 2001; El Khoury et al., 2007). Our lab has previously shown that microglial activation/migration induced by excitotoxic injury is attenuated in MCP1^{-/-} mice (Sheehan et al., 2007). Similar results were found in rats or mice injected with MCP1 blocking antibody (Galasso et al., 2000; Sheehan et al., 2007). Interestingly, like MCP1^{-/-} mice, excitotoxicity-induced microglial activation/migration is decreased in mice lacking plasminogen (plg) or tissue plasminogen activator (tPA), which converts plg to active plasmin (Tsirka et al., 1995; Tsirka et al., 1997). These results indicated that mouse MCP1 and the plg activation system may use converging pathways. Further studies in our lab have revealed that plasmin, generated by the action of tPA on plg in the mouse CNS (or urokinase plasminogen activator in other systems), cleaves MCP1 at lysine (K) 104 (Sheehan et al., 2007). This cleavage removes the highly glycosylated C-terminal extension and generates a N-terminal fragment that is highly homologous to human MCP1. The chemotactic potency of plasmin-cleaved MCP1 is higher than that of intact MCP1 and comparable to human MCP1 (Sheehan et al., 2007; Yao and Tsirka, 2010), suggesting that plasmin is an activator of mouse MCP1. In accordance with this, infusion of plasmin-cleaved MCP1 into the CNS restored excitotoxicity-induced microglial activation/migration in plg^{-/-} mice, whereas infusion of FL-MCP1 failed to do so. Furthermore, we have shown that mouse MCP1 C-terminus, when fused to human MCP1, is also inhibitory (Yao and Tsirka, 2011a). These data indicate that plasmin-mediated cleavage may be a mechanism used by cells to activate MCP1 and initiate downstream signaling cascades in mice. Importantly, the effects of plasmin cleaved MCP1 are comparable to that of human MCP1 (Yao and Tsirka, 2010), suggesting that human MCP1 may be regulated similarly by an unidentified protein or differently at transcriptional/translational, transport, or signaling levels. We favor the latter, because we were not successful in pulling down proteins that specifically interact with human MCP1. In addition,

the two CCR2 isoforms in human (CCR2A and CCR2B) have been shown to be able to activate different signaling pathways. Calcium influx has been found in chemotaxis of CCR2B-positive cells, but not in CCR2A-positive cells (Cho et al., 2007; Sanders et al., 2000), suggesting the MCP1-CCR2 axis may also be regulated at the receptor or signaling levels in human beings.

Although human MCP1 does not have a highly glycosylated C-terminus, it also can be truncated in the C-terminus. A fragment with 69 amino acids (1–69) has been found and this fragment has the same activity as the wild-type MCP1 (Proost et al., 1998). Which enzyme(s) is responsible for this cleavage, however, is not clear. In the N-terminus, human MCP1 has been reported to be cleaved by matrix metalloproteinase-1, -3, -8, and -12 between amino acid 4 and 5 (Dean et al., 2008; McQuibban et al., 2002). This cleavage generates a fragment (5–76) that functions as an antagonist for CCR2 (Dean et al., 2008; Gong and Clark-Lewis, 1995; McQuibban et al., 2002; Proost et al., 1998). Consistently, the MCP1 mutant lacking amino acids 2–8 (7ND) has been shown to inhibit MCP1-CCR2 signaling both *in vitro* and *in vivo* (Kitamoto and Egashira, 2003; Ni et al., 2001; Zhang and Rollins, 1995). There is no report on whether these matrix metalloproteinases cleave mouse MCP1. However, it would be reasonable to assume they do, based on the similarity of human and mouse MCP1 sequence. The first eight amino acids for human and mouse MCP1 are QPDAINAP and QPDAVNAP, respectively.

3.6. Neurons

It is estimated that in human brain every neuron has its own capillary (Zlokovic, 2008), which together with neurovascular coupling (local neuronal activity and metabolism regulate cerebral blood flow) (Iadecola, 2004) indicates that neurons may regulate BBB functions. Anatomical studies have shown that the BMECs and astrocytic processes are directly innervated by noradrenergic, serotonergic, cholinergic, and GABAergic neurons (Ben-Menachem et al., 1982; Cohen et al., 1996; Cohen et al., 1997; Hawkins and Davis, 2005; Tong and Hamel, 1999; Vaucher and Hamel, 1995; Vaucher et al., 2000). The presence of neurons has been shown to increase the integrity of BBB *in vitro* (Minami, 2011). In addition, neurons have been shown to promote the expression of specific enzymes on BMECs (Persidsky et al., 2006). These data suggest that neurons contribute to the integrity of BBB.

MCP1 exerts profound functions on neurons directly and indirectly. MCP1 has been shown to directly bind to CCR2 on rat spinal neurons and activates Akt pathway. The binding of MCP1 on these neurons potently and efficiently inhibits GABA(A)-mediated GABAergic responses (Gosselin et al., 2005). MCP1-CCR2 axis has also been shown to contribute to neuropathic pain via interaction between astrocytes and neurons (Guo et al., 2012; Old and Malcangio, 2012; Zhang et al., 2013; Zhang et al., 2012). Contrary to these reports, MCP1 has been found to protect against methylmercury neurotoxicity (Godefroy et al., 2012) and early expression of MCP1 in neurons is necessary for hypoxic preconditioning induced ischemic tolerance to focal stroke (Stowe et al., 2012). MCP1 induced molecular changes in neurons may modulate the function of other BBB components, and thus affect the integrity of BBB. In accordance with this hypothesis, MCP1 level has been found to positively correlate with the permeability of BBB and progression of disease, including Gaucher's disease, brain inflammation, stroke, and excitotoxic injury (Capoccia et al., 2008; Dimitrijevic et al., 2006; Frangogiannis et al., 2007; Hanisch and Kettenmann, 2007; Kim et al., 2008; Morimoto et al., 2008; Schellenberg et al., 2012; Sheehan et al., 2007; Vitner et al., 2012; Yan et al., 2007; Yao and Tsirka, 2011b). Consistently, lack of MCP1 or CCR2 prevents neuronal death, decreases BBB permeability, and improves neuronal function in

many disorders, including hemorrhage and ischemia-reperfusion injury (Dimitrijevic et al., 2006; Dimitrijevic et al., 2007; Yao and Tsirka, 2012).

4. FUTURE DIRECTIONS

Although it is clear that plasmin removes the C-terminus of mouse MCP1 and enhances its chemotactic activity (Sheehan et al., 2007; Yao and Tsirka, 2010), how human MCP1, which lacks the heavily glycosylated C-terminus, is regulated is still elusive. Future work should focus on the regulation/activation of human MCP1. In addition, how MCP1 regulates individual BBB components, especially pericytes, astrocytes, neurons, and BM, needs investigations. Such studies will shed light on our understanding of MCP1-CCR2 signaling/functioning, and may provide new molecular targets to interfere with many CNS diseases, such as brain trauma, stroke and neurodegenerative diseases right at the level of BBB.

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Figure 1. Proposed model for MCP1-induced BBB compromise

By binding to CCR2, MCP1 induces phosphorylation of ERM proteins, which then bind to ZO-1 and actin cytoskeleton. Additionally, MCP1 also activates Rho associated kinases, which phosphorylate and inactivate MLCP, resulting in increased phosphorylation of MLC. The over-phosphorylation of MLC induces enhanced and prolonged actin-myosin contraction, which generates forces that pull ZO-1 away from the cell-cell border, leading to BBB compromise. Adapted from (Yao and Tsirka, 2011b).