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Molecular insights and therapeutic targets for blood-brain barrier disruption in ischemic stroke: critical role of matrix metalloproteinases and tissue-type plasminogen activator

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Abstract

Blood-brain barrier (BBB) disruption, mediated through matrix metalloproteinases (MMPs) and other mechanisms, is a critical event during ischemic stroke. Tissue plasminogen activator (tPA) is the only FDA-approved thrombolytic therapy for acute ischemic stroke, but the efficacy and safety of its therapeutic application is limited by narrow treatment time windows and side effects. Thus, there is a pressing need to develop combinational therapy that could offset tPA side effects and improve efficacy in clinical practice. Recent experimental studies indicate that tPA has previously unidentified functions in the brain beyond its well established thrombolytic activity, which might contribute to tPA-related side effects through MMPs (mainly MMP-9) and several signaling pathways involved in LDL receptor-related protein (LRP), activated protein C (APC) and protease-activated receptor 1 (PAR-1), platelet-derived growth factor C (PDGF-C), and N-methyl-D-aspartate (NMDA) receptor. Therapeutic targeting of MMPs and/or tPA-related signaling pathways might offer promising new approaches to combination therapies for ischemic stroke. This review provides an overview of the relationship between structural components and function of the BBB/neurovascular unit with respect to ischemic stroke. We discuss how MMPs and tPA contribute to BBB disruption during ischemic stroke and highlight recent findings of molecular signaling pathways involved in neurotoxicity of tPA therapy.

Keywords

Blood-brain barrier; neurovascular unit; tPA; MMPs; ischemic stroke; signaling pathways

Introduction

The blood-brain barrier (BBB) is primarily formed by specialized brain endothelial cells that are interconnected by well-developed tight junctions and provides a dynamic interface between

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the blood and the brain (Abbott, et al., 2010). BBB disruption is a critical event in the pathogenesis of acute ischemic stroke, however, the molecular mechanisms involved are not completely understood (Sandoval and Witt, 2008). Emerging studies indicate that matrix metalloproteinases (MMPs) and tissue-type plasminogen activator (tPA) play critical roles in the BBB disruption during acute ischemic stroke (Adibhatla and Hatcher, 2008). Experimental data suggest that MMPs have deleterious roles in the early phase of ischemic stroke, but also have beneficial roles in stroke recovery during the later phase. By degrading neurovascular matrix and disruption of the BBB tight junctions, MMPs (mainly MMP-9) promote BBB damage, brain edema and hemorrhage during acute ischemic stroke (Sandoval and Witt, 2008). tPA is the only thrombolytic drug approved by the U.S. FDA, but the efficacy and safety of its therapeutic application is limited by narrow treatment time windows (within 3h of the onset of stroke symptoms) and side effects on brain edema and hemorrhagic complications (Cronin, 2010; Derex and Nighoghossian, 2008; Gravanis and Tsirka, 2008). Experimental data have shown that tPA have pleiotropic actions in the brain beyond its well established thrombolytic role, including activation of MMPs and other molecular pathways (Adibhatla and Hatcher, 2008; Yepes et al., 2009; Rosell and Lo, 2008). These effects may increase tPA neurotoxicity, further damage the BBB, and worsen edema and cerebral hemorrhage (Adibhatla and Hatcher, 2008; Yepes et al., 2009). Thus, combination therapies targeting MMPs and other tPA-related pathways may limit neurotoxic effects and extend treatment time windows of tPA in ischemic stroke. This review provides an overview of the relationship between structural components and function of the BBB with respect to ischemic stroke. We discuss how MMPs and tPA contribute to BBB disruption during ischemic stroke and highlight recent findings of molecular signaling pathways involved in neurotoxicity of tPA therapy.

1. The blood-brain barrier structural component and functional integrity

The structure and function of the blood-brain barrier (BBB) has been discussed in reviews elsewhere (Sandoval and Witt, 2008; Abbott, et al., 2010). Structural and molecular components of the BBB are summarized in Fig. 1. Briefly, the BBB comprises the tight junctions (TJs) and adherens junctions (AJs). TJs are continuous membrane strands located at the apical site between brain endothelial cells (ECs), which consist of three integral protein types: claudins, occludin, and junctional adhesion molecules (JAMs) (Abbott et al., 2010). TJs also consist of several accessory proteins, including zonula occludens (ZO), cingulin, 7H6, AF-6, and others (Abbott, et al., 2010). These accessory proteins may serve as recognition proteins for tight junctional placement and as a support structure for signal transduction proteins (Hawkins and Davis, 2005; Piontek et al., 2008). While not traditionally considered as a TJ protein, the actin cytoskeleton in brain ECs plays a critical role in modulating BBB permeability (Lai et al., 2005).

Claudins are small tetraspan membrane proteins (20–24 kDa) with two extracellular loops that form dimers and are able to bind homotypically to adjacent endothelial cells to form the primary seal of the tight junctions (Furuse et al., 1999; Piontek et al., 2008). Several claudin family members, such as claudins-3, -5, and -12, have been identified in BBB endothelial cells (Sandoval and Witt, 2008; Hawkins and Davis, 2005). Among the claudin family members, claudin-5 has been shown to be a major cell adhesion molecule of BBB tight junctions (Nitta et al., 2003). Occludin is a 60–65 kDa tetraspan membrane protein that is exclusively localized at TJ strands (Sandoval and Witt, 2008; Hirase et al., 1997). Occludin's carboxy-terminal binds to zonula occludens (ZO) proteins, which in turn binds to the actin cytoskeleton (Haskins et al., 1998; Fanning et al., 1998; Liu et al., 2008). Although occludin is not required for the formation of TJ strands per se, the presence of occludin in the membrane is associated with increased electrical resistance across the membrane and decreased paracellular permeability (Balda et al., 1996; Saitou et al., 2000; Tsukita et al., 1999). Molecular structure and function of TJ proteins can be regulated by alterations in their expression levels and/or distribution at

the BBB under various pathophysiological conditions, including ischemic stroke (Sandoval and Witt, 2008). Experimental studies suggest that phosphorylation represents a critical mechanism regulating distribution/localization and function of BBB TJ proteins (Yamamoto et al., 2008; Feldman et al., 2005). For example, phosphorylation of claudin-5 at Thr207, via the protein kinase-A (PKA) (Soma et al., 2004) and Rho kinase activation (Yamamoto et al., 2008), has been shown to increase BBB permeability. Tyrosine phosphorylation of occludin has been identified with its disassociation from intracellular ZO proteins and increased BBB permeability (Rao et al., 2002; Kale et al., 2003; Kago et al., 2006). More such efforts are needed to better understand how TJ proteins are phosphorylated at the level of specific residues (e.g. serine/threonine/tyrosine) and how their phosphorylation differentially and synergistically contributes to BBB disruption at different stages of stroke.

JAMs are a family of immunoglobulin superfamily proteins (~40 kDa) that localize within the intercellular cleft between brain ECs (Abbott et al., 2010). Heretofore, three JAMs (-A, -B, and –C) have been identified in brain ECs (Abbott et al., 2010). Intriguingly, JAMs have direct inflammatory-related activities because they are also expressed in leukocytes and platelets (Weber et al., 2007). JAM-A is required for neutrophil infiltration in inflammatory or ischemic tissues by controlling beta1-integrin internalization and recycling (Cera et al. 2009). Anti-JAM-A monoclonal antibody (BV11) reduces monocyte transmigration across endothelial cells in vitro and in vivo (Del Maschio et al., 1999; Martin-Padura et al., 1998). JAM-B and -C have an impact on leukocyte adhesion and migration. JAM-B can bind to integrin VLA-4 (α4β1), a leukocyte integrin that contributes to rolling and firm adhesion of lymphocytes to endothelial cells through binding to vascular cell adhesion molecule (VCAM)-1, but this effect requires prior engagement with JAM-C (Cunningham et al., 2002; Ludwig et al., 2009). JAM-C can act as a novel counterreceptor for the integrin Mac-1 (CD11b/CD18) and mediates leukocyte transmigration independently of JAM-B (Santoso et al., 2002; Keiper et al., 2005; Chavakis et al., 2004). Taken together, experimental data suggest that JAMs may play an active role in the regulation of cerebral inflammatory response and BBB permeability during ischemic stroke.

The adherens junctions (AJs) are mainly composed of VE-cadherin (vascular endothelial cadherin), which are necessary for formation of TJs by forming a continuous belt localized near the apical end of the junctional cleft, just below the TJs (Dejana et al., 2008). While not considered as the primary BBB paracellular barrier, AJs are functionally and structurally linked to TJs and may play an important role in the localization and stabilization of the TJs (Taddei et al., 2008). Experimental data show that endothelial VE-cadherin at AJs upregulates TJ protein claudin-5, suggesting a direct regulation of TJ integrity by AJ proteins (Taddei et al., 2008).

2. Expression, activation, and function of MMPs in BBB disruption in ischemic stroke

Matrix metalloproteinases (MMPs) comprise a family of zinc endopeptidases that can broadly target almost all components of the mammalian central nervous system (CNS). Emerging evidence indicates that MMPs play both detrimental and beneficial roles in ischemic stroke. In the early phase (hours to days) after cerebral ischemia, MMPs disrupt the BBB by degrading the TJ proteins (e.g. occludin and claudin-5) and basal lamina proteins (e.g. fibronectin, laminin, collagen, proteoglycans, and others) and thereby lead to BBB leakage, leukocyte infiltration, brain edema, and hemorrhage (Adibhatla and Hatcher, 2008; Cunningham et al., 2005). In contrast, MMPs may play beneficial roles in stroke recovery by modulating neurovascular remodeling (Cunningham et al., 2005; Zhao et al., 2006).

2.1 Expression and activation of MMPs in the brain during ischemic stroke—

Expression of MMPs in the adult brain is very low to undetectable under normal conditions.

Clinical and experimental studies have demonstrated that several MMPs such as MMP-2, MMP-3, MMP-7, or MMP-9 are upregulated and activated after ischemic stroke (Clark, et al., 1997; Rosell et al., 2006; Heo et al., 1999; Anthony et al., 1997; Suzuki et al., 2007; Sole et al., 2004). In the brain, MMPs can be expressed by various cell types, including resident cells (endothelial cells, microglia, neurons, and astrocytes) and infiltrating inflammatory cells during cerebral ischemia, but the brain regions and cellular sources of expression differ according to the specific MMPs, as well as the type, severity and duration of injuries (Adibhatla and Hatcher, 2008; Cunningham et al., 2005; Jin et al., 2010). Experimental data indicate that brain microvascular ECs and infiltrating leukocytes (most likely neutrophils) are key cellular sources of brain MMP-9 at least in the early phase (within 24h) after focal cerebral ischemia (Jin et al., 2010; Justicia et al., 2003; Gidday et al., 2005; McColl et al., 2008). By immunostaining and microdissection, clinical data confirm that microvessel endothelium and infiltrating neutrophils are the major source of the increased brain MMP-9 after ischemic and hemorrhagic stroke in humans (Rosell et al., 2006 & 2008b).

MMPs are synthesized and secreted as inactive pro-enzymes that subsequently become proteolytically cleaved and activated. To achieve optimal enzymatic activity, MMPs are tightly controlled at the transcriptional level, as well as at the protein level through activation by their zymogens and inhibition by tissue-specific inhibitors (Cunningham et al., 2005). Potential mechanisms contributing MMP activation are summarized in Fig. 2. During cerebral ischemia, proMMP-2 can be activated by membrane-type MMP (MT1-MMP), and the latter is activated by furin (Candelario-Jalil et al., 2009; Yang et al., 2007), and proMMP-9 can be activated by MMP-3 (stromelysin-1), as well as other mechanisms such as proinflammatory factors (e.g. IL-1β, TNF-α, CD40L, and many others) and reactive oxygen species (ROS) (Gasche et al., 2001; Haorah et al., 2007; Jian et al., 2005). tPA has been shown to activate MMPs through plasmin-dependent and –independent mechanisms (Candelario-Jalil et al., 2009). In the brain, the molecular mechanisms by which MMPs are upregulated and activated during ischemic stroke are not fully understood. It remains largely unclear how the expression and activation of specific MMPs are differentially regulated in different cell types within the neurovascular unit.

The neurovascular unit is comprised of the endothelial cells which make up the vessels as well as perivascular neurons, astrocytes, and pericytes (del Zoppo, 2006) (Fig. 3). Over the past decade, an extensive investigation of the BBB in cerebrovascular disease has expanded from consideration of only endothelial cells to include interactions with different types of cells and extracellular matrix in the neurovascular unit. In recent years, the concept of the neurovascular unit has emerged as a new paradigm for stroke investigation and therapy. This concept emphasizes that cell-cell signaling among the various neuronal, glial, and vascular compartments underlies the homeostasis of normal brain function (Guo and Lo, 2009). Conversely, dysfunctional signaling within the neurovascular unit should contribute to disease. There are temporal and spatial changes of MMP-9 within the cells of the neurovascular unit after stroke (Zhao et al., 2006). In a rat model of transient middle cerebral artery occlusion (MCAO), most of the MMP-9 activities colocalized with brain microvessel ECs within 24 hours, but at 7 to 14 days, the MMP-9 signal shifted to the periphery of cortical infarction and was associated mainly with neurons and astrocytes (Zhao et al., 2006). This redistribution within the neurovascular unit likely reflects multiphasic roles of MMP-9 in ischemic stroke, that is, the pathological role in mediating disruption of the BBB, neuronal cell death and hemorrhage early after stroke, and the beneficial role in mediating neurovascular remodeling during the repair phase (Adibhatla and Hatcher, 2008; Zhao et al., 2006; Cunningham, et al., 2005). Inhibition of MMP-9 during the late phase (7 to 14 days) after stroke has been shown to reduce the number of neurons and new vessels that correlated with increased brain injury and impaired functional recovery (Zhao et al. 2006). Thus, it is reasonable to speculate that

blocking MMPs at a badly chosen time and in nontarget cell types may result in unwanted side effects (Zlokovic, 2006).

2.2 Biphasic opening of BBB during ischemic stroke—BBB opening during focal cerebral ischemia/reperfusion (I/R) injury has long been considered to follow a biphasic time course, but considerable discrepancies across studies exist with respect to the timing of the second opening (Belayev et al., 1996; Huang et al., 1999; Kuroiwa et al., 1985; Sharp et al., 2000; Chen et al., 2009a). Morphologically, BBB opening correlates with a redistribution of the TJ and AJ proteins from the plasma membrane to the cytoplasm as well as reorganization of the endothelial actin cytoskeleton (Bolton et al., 1998; McColl et al., 2007; McColl et al., 2008). The extent of BBB disruption is associated with the type and severity and duration of ischemic insults. A mild to moderate opening of the BBB may be partially reversible, and allows plasma constituents to enter brain and possibly damage cells. In contrast, severe BBB disruption after ischemic stroke is unlikely to be reversible and allows even further extravasation of potentially harmful plasma constituents (Chen et al., 2009a). The molecular mechanisms underlying BBB opening and its consequences in ischemic stroke are not fully understood. Several MMPs (especially MMP-9) have been implicated in the regulation of BBB permeability and function during ischemia stroke.

2.3 Differential roles of MMP-2 and MMP-9 in BBB disruption during ischemic stroke—Among MMPs, MMP-2 and MMP-9 are two of the most widely studied enzymes that have been shown to be critical in regulating BBB permeability during cerebral ischemia. The two enzymes appear to differentially mediate disruption of the BBB and neuronal damage after cerebral ischemia. MMP-2 gene knockout does not provide neuroprotection in mouse models of permanent and transient MCA occlusion (Asahi et al., 2001a). Consistently, in vitro data show that MMP-2 is not toxic to neurons in hippocampal slice preparations (Cunningham, et al., 2005). In contrast, MMP-9 gene knockout provides strong neuroprotection in the same animal models, and in vitro MMP-9 is toxic to neurons in hippocampal slice preparations and in cultured primary cortical neurons (Asahi et al., 2000; Asahi et al., 2001b). In accordance with these findings, a recent clinical study (Lucivero et al., 2007) suggests that MMP-9 and MMP-2 may play different roles in human ischemic stroke. Increase in plasma MMP-2 is observed only in patients with lacunar (mild) stroke early (within 12 h) and related to better outcome. In contrast, increase in plasma MMP-9 seems to be late (at day 7) and related to more severe stroke.

Earlier experimental studies provided indirect evidence that MMP-2 played a key role in the initial opening of the BBB after cerebral ischemia. In a rat model of transient middle cerebral artery occlusion (MCAO), the initial opening at 3 hours correlated with brain MMP-2 levels and was blocked by a synthetic MMP inhibitor (BB-1101) (Rosenberg, et al., 1998). The second, delayed opening (maximal opening at 48 hours) appeared to correlate with brain MMP-9 levels (maximally elevated at 48 hours), but had no response to the MMP inhibitor (Rosenberg, et al., 1998). Increased expression of MMP-2 may contribute to the initial opening of BBB by degrading the basal lamina leading to neuronal injury (Heo et al., 1999; Chang et al., 2003). In the non-human primate brains, MMP-2 increased significantly as early as 1 hour after transient MCAO and was persistently elevated at least 7 days (Heo et al., 1999; Chang et al., 2003). Direct injection of MMP-2 into the rat brain resulted in the disruption of the BBB with subsequent hemorrhage, and this effect was inhibited by co-administration of TIMP-2 (Rosenberg, et al., 1992). A recent study (Yang et al., 2007) suggests a potential mechanism whereby MMPs mediate the BBB disruption during ischemic stroke. In a rat model of transient MCAO, the initial opening of the BBB occurred as early as 3 hours after reperfusion and increased activation of MMP-2 correlated with the early opening of the BBB. Correspondingly, the mRNA expression of claudin-5 and occludin, decreased in both hemispheres, and both proteins degraded or fragmented in ischemic hemispheres after 2–3 h of reperfusion, and

treatment with the MMP inhibitor BB-1101 reversed the degradation of the TJ proteins. Thus, the early degradation of the TJ proteins seems to be associated with a marked increase in MMP-2 in the early phase after cerebral ischemia. Experimental data clearly demonstrate greater increase in MMP-2 than in MMP-9 at 3 h, along with increased expression of MMP-2 activators, MT1-MMP and furin. In contrast, there was no increase in MMP-2 mRNA and activity, while MMP-9 mRNA and activity markedly increased at 24 h of reperfusion, suggesting an association between MMP-9 and delayed disruption of the BBB. Immunostaining shows that claudin-5 and occludin, though loosened or fragmented, remained within the endothelial clefts during the initial opening of the BBB. Together, these data suggest that the initial opening is a reversible, which is regulated, at least in part, by the activation of MMP-2.

Recent studies suggest that MMP-9 plays a critical role in mediating the second, delayed opening of BBB after ischemic stroke. Emerging data indicate that MMP-9 is associated with severe BBB disruption by further degrading the tight junctions and basal lamina proteins, substantially contributing to brain infarction, edema, and hemorrhagic transformation (HT) in both animal models (Yang, et al., 2007; Lee et al., 2007; Rosenberg, et al., 2007) and in human stroke patients (Rosell et al., 2008; Montaner et al., 2003; Barr, et al., 2009). MMP-9−/− mice display a significant reduction in BBB disruption and brain edema and this effect is associated with reduced degradation of intracellular ZO-1 as compared to wild-type mice after transient MCAO (Asahi, et al., 2001b). MMP-9 has been shown to degrade TJ proteins (claudin-5, occludin, ZO-1) in cultured brain ECs (Chen et al., 2009b) and in animal models of focal cerebral ischemia (Yang et al., 2007; McColl et al., 2008; Bauer et al., 2009; Liu et al., 2009).

There are a few controversies concerning the time course of the expression and activation of MMP-2 and MMP-9 in the brain after ischemic stroke. Some experimental data suggest that MMP-9 responses appear to dominate in the acute phase, whereas MMP-2 elevations seem to occur in the delayed phase after stroke. In the rat transient MCAO model, Planas et al. (2001) shows that MMP-9 is induced and activated from 4 h to 4 days, but a small increase in MMP-2 is detected at 4 h, while a massive increase in MMP-2 expression and activation by day 4. In the mouse permanent MCAO model, Gasche et al. (1999) shows that expression and activation of MMP-9 in the ischemic brains are induced significantly as early as 2–4 h after ischemia, with increased BBB permeability. In contrast, the pro-MMP-2 is induced significantly only after 24 h of permanent ischemia, and no activated form is observed (Gasche et al., 1999). These data suggest that MMP-9, but not MMP-2, plays an active role in early BBB disruption after ischemic stroke.

Recent studies suggest that MMP-9 may play a more prominent role in the BBB disruption during ischemic stroke under clinical relevant conditions linked to elevated systemic inflammation. Experimental data have shown that systemic inflammation exacerbates neutrophil infiltration into the ischemic brain and thus alters the kinetics of the BBB tight junction disruption after experimental stroke (McColl et al., 2007 & 2008). These studies demonstrate that infiltrating neutrophils are the primary source of increased (5-fold) MMP-9 activity in the ischemic brain of mice challenged with interleukin-1β (IL-1β) at 4, 8 or 24h after focal cerebral ischemia. A transformation from transient to sustained BBB disruption caused by enhanced neutrophil-derived MMP-9 is a critical mechanism underlying the exacerbation of ischemic brain injury by IL-1β-induced systemic inflammation, which is mediated through conversion of a transient to a sustained disruption of the TJ protein (claudin-5) and exacerbated disruption of the cerebrovascular basal lamina protein (collagen-IV) (McColl et al., 2008). This mechanism may contribute to the poor clinical outcome in stroke patients with elevated systemic inflammatory status. In stroke patients with prior infection and atherosclerosis, neutrophil infiltration into the ischemic brain and neutrophil-

derived neurovascular MMP-9 are elevated (Buck et al., 2008; Emsley et al., 2003; Zeller et al., 2005).

Taken together, the above findings support the hypothesis that MMP-2 and MMP-9 play important but distinct roles in mediating BBB disruption after ischemic stroke. MMP-2 is preferentially associated with the initial opening of the BBB, while MMP-9 appears to be more important in the delayed BBB damage by mediating a conversion from transient to sustained BBB disruption, in particular associated with elevated systemic inflammation.

In addition to MMP-2/-9, other MMP members may also play active roles in mediating BBB disruption during ischemia stroke. MMP-3 (stromelysin-1) has been shown to mediate BBB opening during neuroinflammation (Gurney et al., 2006). After intracerebral injection of lipopolysaccharide (LPS), MMP-3 knockout mice showed less degradation of the TJ proteins (claudin-5, occludin, laminin-alpha-1) together with reduced neutrophil infiltration, compared with wild-type mice (Gurney et al., 2006). In the rat transient MCAO model, brain MMP-3 is activated as determined by the cleavage of the cerebral matrix agrin, an MMP-3 substrate (Sole et al., 2004). It has been speculated that various MMPs function in a network-like fashion with upstream and downstream proteases being closely coupled (Rosell and Lo, 2008). Future studies need to assess not just MMP-2, -3 and -9, but all family members to truly ‘fingerprint’ the role of various MMPs in ischemic stroke (Rosell and Lo, 2008).

3. Expression and neurotoxicity of tPA in BBB disruption during ischemic stroke

Thrombolytic therapy with tissue-type plasminogen activator (tPA) for ischemic stroke represents a two-edged sword, since tPA promotes desirable (thrombolytic) as well as undesirable (neurotoxic) outcomes during stroke (Adibhatla and Hatcher, 2008). It has been established that exogenous tPA can cross both the intact and the damaged BBB to reach the brain parenchyma (Adibhatla and Hatcher, 2008; Yepes et al., 2009). Thus, both endo- and exogenous tPAs are capable of influencing brain functions and dysfunctions. In addition to its intended role in thrombolysis, tPA also possesses important signaling and protease actions in the neurovascular unit after stroke, some of which may mediate neurotoxicity and hemorrhagic transformation (HT) after tPA therapy. Molecular mechanisms underlying tPA’s neurotoxicity within the neurovascular unit have not been fully understood. Better understanding of the activities and neurotoxicity of tPA in the brain may provide a molecular basis for developing effective and safe tPA combination therapy for acute ischemic stroke.

3.1 Expression and regulation of tPA in systemic circulation and in brain—

tPA is a highly specific serine proteinase and is found predominantly in the blood, where its primary function is as a thrombolytic enzyme and its principal substrate is the zymogen plasminogen (Gravanis and Tsirka, 2008). The catalytic activity of tPA is rapidly inactivated in the blood stream through binding of serine protease inhibitor(s), primarily plasminogen activator inhibitor-I (PAI-1). The balance between t-PA and PAI-1 regulates the systemic fibrinolytic potential in the vasculature. The tPA/PAI-1 complex is cleared from the circulation by the liver *via* a scavenging receptor, the low-density lipoprotein receptor-related protein-1 (LRP-1) (Yepes et al., 2003). By the LRP-mediated clearance, tPA has a 5 to 10 min short half-life in the bloodstream in humans (Gravanis and Tsirka, 2008). In the brain, tPA has been identified mainly in the endothelial cells of the BBB and in the endothelium of the small vessels (Levin and del Zoppo, 1994), where it may regulate BBB permeability and vascular tone. tPA is also expressed and released by neurons and microglia and mediates neuronal death and microglial activation after excitotoxic injury (Siao et al., 2003). tPA is synthesized by neurons and glia in most brain regions, particularly abundant in the hippocampus, hypothalamus, cerebellum, and amygdala (Salles et al., 2002; Sappino et al., 1993). A wide distribution of tPA biosynthesis in the brain is likely to be associated with different actions of tPA, such as promoting synaptic

plasticity and regulating the permeability of the neurovascular unit related to cerebral ischemia and seizures (Qian et al., 1993; Seeds et al., 1995; Yepes et al., 2002; Mataga et al., 2002).

In the brain, tPA activity is regulated by neuroserpin, a potent endogenous inhibitor of tPA. Neuroserpin, a member of the serpin (serine proteinase inhibitors) family, is expressed primarily in the brain, where it reacts preferentially with tPA by inhibiting its activity (Miranda et al., 2006). Using transgenic mice overexpressing (~6-fold) neuroserpin in the nervous system, Cinelli et al. (2001) demonstrates that neuroserpin reduces microglial activation and, therefore, the tPA activity and has a neuroprotective role in mice after focal ischemic stroke. In rodent experimental cerebral ischemia, Lebeurrier et al. (2005) demonstrates that administration of exogenous neuroserpin into the brain protects neurons against NMDA-induced excitotoxicity, calcium influx and tissue damage in vitro and in vivo. Together, these data suggest that overexpression or administration of neuroserpin in the brain may limit the deleterious effects of tPA after cerebral ischemia. Indeed, adjuvant treatment with neuroserpin has been shown to significantly reduce BBB leakage, brain edema, and infarct size and extend the therapeutic window for tPA administration in a rat model of embolic stroke (Zhang et al., 2002).

3.2 Neurotoxicity of the tPA-MMP-9 (and MMP-3) pathway during ischemic stroke—The standard hypothesis postulates that MMP-9 play a central role in tPA-mediated neurotoxicity in thrombolytic therapy for acute stroke (Rosell and Lo, 2008). This hypothesis is supported by data from both clinical and experimental studies. Clinical data have shown that plasma and brain levels of MMP-9 are elevated in patients with acute ischemic stroke, and delayed (>3 h of symptoms onset) tPA therapy causes a further increase in plasma MMP-9 in stroke patients (Barr et al., 2010; Castellanos et al., 2003; Castellanos et al., 2007; Horstmann et al., 2003; Kelly et al., 2008; Montaner et al., 2003; Rosell et al., 2006; Rosell et al., 2008b). MMP-9 in blood has emerged as a promising biomarker for human stroke, because plasma levels of MMP-9 correlate with and predict poor neurological outcome and hemorrhagic complications after thrombosis in these clinical trials.

Experimental studies provide direct evidence supporting the tPA-MMP-9 hypothesis. In rodent stroke models, endogenous tPA activity in the brain is induced as early as 1 h (preceding changes in MMP-9 and BBB integrity) after focal cerebral ischemia and is present mainly in the perivascular tissue and vessel wall (Tsuji et al., 2005; Yepes et al., 2003). Genetic deficiency of tPA or inhibition of its activity by neuroserpin has been shown to decrease BBB disruption, edema, neuronal death and brain infarction (Tsuji et al., 2005; Wang et al., 1998). Further, examination of both tPA−/− and wild-type (WT) mice demonstrates that the increased endogenous tPA is required for the initial opening of the BBB after transient MCAO (Yepes et al., 2003). Levels of MMP-9 in ischemic brain tissues are significantly reduced in tPA−/− mice compared with wild-type mice and intravenous administration of exogenous tPA in tPA −/− mice can rebuild the ischemic MMP-9 response back up to WT levels (Tsuji et al., 2005; Wang et al., 1998). Intravenous administration of exogenous tPA also increases MMP-9 levels in ischemic brains in rats after transient MCAO (Yepes et al., 2003). Together, these findings support the hypothesis (Fig. 2) that tPA is a major upstream mediator of MMP-9 induction during ischemic stroke, especially when tPA is exogenously administrated.

Although numerous studies have established the neurotoxicity of tPA in ischemic stroke, some studies demonstrate show neurotorphic effects of tPA on neurons, independent of thrombolysis. An earlier study by Kim et al. (1999) shows that recombinant tPA markedly attenuated zinc-induced neuronal cell death in cortical culture, and, when injected into cerebrospinal fluid, also reduced kainate seizure-induced hippocampal neuronal death in adult rats. A recent study (Lee et al., 2007) by the same group shows that low dose tPA (500 ng/ml) treatment induces neurotrophic effects, promoting neurite elongation and neuronal survival in cortical neuronal

cultures, and this neurotrophic effect is mediated by typical trophic signaling kinases such as Raf-K/ERK, PKC, and PI3-K/Akt, but not mediated by its proteolytic action because it is not affected by tPA protease inhibitors, such as PAI-1. In contrast, high-dose tPA (20ug/ml) has been shown to induce neurotoxicity in cortical cultures (Nocole et al., 2001). Nevertheless, it remains unclear if similar effects take place in vivo during ischemic stroke, especially when treated with tPA at therapeutic doses (e.g. 2.5–10mg/kg).

In addition to MMP-9, other MMP members may also play active roles in tPA-mediated neurotoxicity in thrombolytic therapy for acute stroke. A recent study (Suzuki et al., 2007) shows that MMP-3 is critical for intracranial bleeding after t-PA treatment of stroke in mice. MMP-3 expression is significantly enhanced in the ischemic hemisphere, where it is expressed only in neurons, but its expression is up-regulated in endothelial cells with t-PA treatment. MMP-3 knockout mice treated with tPA display significantly reduced hemorrhagic transformation (HT) than wild-type mice treated with tPA. In vitro, t-PA induces MMP-3 in cultured murine brain ECs, and this effect is prevented by inhibition of either LDL receptor-related protein (LRP) or NF-kB activation (Suzuki et al., 2009). Collectively, experimental data indicate that tPA enhances brain MMP-9 (and MMP-3) levels in stroke in vivo, and suggest that combination therapies targeting MMPs may improve tPA therapy.

3.3 Molecular signaling pathways associated with tPA neurotoxicity

The tPA-LRP pathway: The LDL receptor-related protein (LRP) is a member of the LDL receptor gene family that binds several ligands, including tPA (Herz and Strickland, 2001). In the brain, LRP is found in neurons and in perivascular astrocytes (Polavarapu et al., 2007) [134], and the interaction between tPA and LRP has been shown to regulate cerebrovascular tone (Nassar et al., 2002) and BBB permeability (Yepes et al., 2003; Polavarapu et al., 2007). Increased tPA activity in the ischemic brain has been associated with worsening the ischemic lesion (Wang et al., 1998; Yepes et al., 2000), neuronal death (Tsirka et al., 1995; Tsirka et al., 1997), and BBB disruption (Yepes et al., 2003; Polavarapu et al., 2007). Injection of recombinant tPA into the cerebrospinal fluid in the absence of ischemia results in a rapid dose-dependent increase in the BBB permeability (Yepes et al., 2003), and this effect is inhibited by blocking LRP using anti-LRP antibodies or the LRP antagonist called the receptor-associated protein (RAP), suggesting the tPA's action is mediated via its receptor LRP (Yepes et al., 2003).

After acute ischemic stroke, the thrombolytic action of tPA in the intravascular space is beneficial, whereas its effect on neurons in the extravascular space is considered deleterious (Adibhatla and Hatcher, 2008; Yepes et al., 2009; Zhang et al., 2002). It has been postulated that if tPA therapy effectively reverses ischemia promptly and the BBB remains intact, then (exogenous) tPA remains within the vascular space. In contrast, if tPA therapy is ineffective and ischemia is prolonged, then there is the risk that exogenous tPA will across BBB and enter the brain parenchemia and thereby damage both the brain and the neurovascular unit by interacting with LRP (Adibhatla and Hatcher, 2008; Yepes et al., 2009). Indeed, some studies have demonstrated that tPA may cross the intact BBB by LRP-mediated transcytosis (Benchenane et al., 2005) and enter the brain from the intravascular space under ischemic and nonischemic conditions (Benchenane et al., 2005a and 2005b). Desmoteplase, a recombinant form of the plasminogen activator from saliva of the vampire bat *Desmodus rotundus*, has been shown to antagonize vascular tPA-induced neurotoxicity possibly by competing with tPA for the LRP binding at the BBB and thus effectively blocking tPA access to the brain parenchyma (López-Atalaya et al., 2007). Desmoteplase might offer a safe, effective adjunct therapy for tPA in acute stroke, since it is not neurotoxic and has longer (> 4 hr) half-life in circulation compared to tPA (5–10 min) (López-Atalaya et al., 2007).

Experimental studies have shown that LRP signaling plays an important role in the tPA-induced expression and activation of MMP-9 (and MMP-3) in cell culture and in animal models of stroke (Suzuki et al., 2009; Wang et al., 2003; Yepes et al., 2003). In vitro, recombinant tPA treatment stimulates MMP-9 expression in cultured human brain ECs, and this tPA-induced response is significantly reduced in the ECs treated with siRNA to suppress LRP, and was absent in LRP-deficient MEF cells (Wang et al., 2003). In vivo, direct intraventricular injection of tPA into mouse brain increases BBB permeability and this response is attenuated by LRP antagonists (Yepes et al., 2003). In addition, tPA has been shown to induce MMP-3 (stromelysin-1) in cultured murine brain ECs and this induction by t-PA is prevented by inhibition either of LRP or of nuclear factor (NF)-κB activation (Suzuki et al., 2009). These findings indicate that t-PA promotes BBB damage via MMP-9 (and MMP-3) induction in endothelial cells, which is regulated through the LRP/NF-κB pathway (Fig. 2). Moreover, the tPA-LRP interaction has been shown to induce the expression of iNOS in astrocytes during cerebral ischemia via the activation of the NF-κB pathway (Zhang et al., 2007), which might contribute to increased oxidative stress and BBB damage in ischemic stroke. Accordingly, targeting the tPA-LRP signaling pathway in brain may offer new approaches for decreasing tPA neurotoxicity and improving stroke therapy (Wang et al., 2003).

The tPA-APC/PAR1 pathway: Activated protein C (APC) is a serine protease with anticoagulant, anti-inflammatory and antiapoptotic activities. Experimental studies indicate that APC is neuroprotective during transient cerebral ischemia and promotes activation of antiapoptotic mechanisms in brain cells by acting directly on endothelium and neurons (Bernard et al., 2001; Cheng et al., 2003 & 2006; Feistritzer et al., 2005; Guo et al., 2004; Liu et al., 2004; Thiyagarajan et al., 2008; Zlokovic et al., 2005). In vitro, tPA substantially increases caspase-3 and caspase-8 activity in mouse cortical neurons treated with NMDA, but did not enhance caspase-9 activity. APC inhibits tPA-induced activation of caspase-8 and caspase-3 in human brain ECs and caspase-3-dependent nuclear translocation of apoptosis-inducing factor (AIF) in NMDA-treated neurons (Liu et al., 2004). In hypoxic human brain ECs treated with tPA, blockade of caspase-8, but not MMP-9, effectively inhibited caspase-3 activation, in contrast, in the absence of tPA, blockade of caspase-9, but not MMP-8, effectively inhibited caspase-3 activation. These findings suggest that tPA shifts the apoptotic pathways from the initiator caspase-9 to caspase-8. Both caspase-8 and caspase-3 activation in neurons treated with the combination of NMDA and tPA are substantially reduced (>80%) by APC. In vivo, recombinant APC can markedly reduce tPA-induced cerebral ischemic injury in a mouse model of transient brain ischemia (Liu et al., 2004) [150]. Further, experimental studies indicate that late APC administration (at 6–72 h or 72–144 h post ischemia) is also neuroprotective and mediates brain repair (i.e., neovascularization and neurogenesis) in a mouse model of transient brain ischemia (Thiyagarajan et al., 2008) [146]. These data suggest a significant extension of the therapeutic window for APC intervention in postischemic brain. Moreover, APC is an FDA-approved drug for severe sepsis. APC stabilizes vascular endothelial barriers (Thiyagarajan et al., 2008; Feistritzer et al., 2005) and has a low risk for brain hemorrhage in patients with severe sepsis (Bernard, et al., 2001) and in animal models of stroke (Cheng et al., 2003; Liu et al., 2004; Zlokovic et al., 2005). Experimental studies have shown that APC promotes neovascularization and neurogenesis in postischemic brain and blocks tPA vascular and neuronal toxicities in vitro and in vivo via protease-activated receptor 1 (PAR-1) (Thiyagarajan et al., 2008; Feistritzer et al., 2005; Cheng et al., 2006). For example, Cheng, et al (Cheng et al., 2006) demonstrate that APC inhibits tPA-induced, NF-κB-dependent MMP-9 pathway in ischemic brain endothelium in vivo and in vitro by acting through PAR-1 (Fig. 2). Taken together, these findings suggest that the combination therapy with tPA and APC may hold a great promise for blocking tPA's neurovascular toxicity and promoting stroke recovery.

The tPA-PDGF-CC pathway: Platelet-derived growth factor C (PDGF-C) is one of four members in the PDGF family, which are known mitogens and survival factors for cells of

mesenchymal origin. Experimental data show that tPA is both necessary and sufficient to directly induce opening of the BBB (Yepes et al., 2003), but this effect is independent of plasminogen, implicating other substrate(s) for tPA. Recently, PDGF-CC has been identified as a new substrate for tPA (Fredriksson et al., 2004 & 2005). tPA can activate and cleave latent PDGF-CC by directly interacting with the N-terminal CUB domains in PDGF-CC (Fredriksson et al., 2005; Li et al., 2000). Experimental data show that PDGF-CC is a downstream substrate of tPA within the neurovascular unit (Su et al., 2008). Activation of PDGF-CC by tPA impairs BBB integrity during ischemic stroke. Intracerebral injection of recombinant tPA or active PDGF-CC even in the absence of ischemia results in a significant increase in BBB permeability (Su et al., 2008). In contrast, co-injection of neutralizing antibodies to PDGF-CC with tPA blocks this increased permeability, indicating that PDGF-CC is a downstream substrate of tPA within the neurovascular unit (Su et al., 2008). Further, data show that the interaction between tPA and PDGF-CC is mediated through activation of PDGF-alpha receptors (PDGFR-α) on perivascular astrocytes, and treatment of mice with the PDGFR-alpha antagonist imatinib reduces BBB permeability and hemorrhage associated with late administration of tPA after ischemic stroke (Su et al., 2008). Taken together, these findings demonstrate that the tPA-PDGFR-α/PDGF-CC pathway regulates BBB permeability and suggest potential new therapeutic strategies for decreasing tPA neurotoxicity and improving stroke therapy.

The tPA-NMDA receptor pathway: The N-methyl-D-aspartate (NMDA) receptor, a glutamate receptor, is the predominant molecular device for controlling synaptic plasticity and memory function. Glutamate is the main excitatory neurotransmitter in the brain. Glutamate levels increase dramatically in cerebral ischemia and stroke. This may lead to opening of the BBB and induce further brain damage. The mechanisms of glutamate-induced disruption of the BBB integrity are not fully understood. Experimental data show that the NMDA receptors are involved in glutamate-induced alterations of the TJ molecule occludin expression and phosphorylation in brain ECs (András et al., 2007). Treatment with glutamate increases tyrosine phosphorylation and decreases threonine phosphorylation of occludin. Inhibition of the NMDA receptors by MK-801 partially protects against glutamate-induced elevation of occludin tyrosine phosphorylation. Inhibition of the NMDA receptors also attenuates glutamate-induced changes in occludin redistribution but not in the total protein levels (András et al., 2007). These findings suggest that glutamate-induced occludin phosphorylation and redistribution, leading to disruption of the BBB functions are regulated by the NMDA receptors.

Several studies have highlighted a role for tPA in modulating glutamatergic neurotransmission through a direct interaction with NMDA receptors, findings suggesting a potential mechanism directly involved in the ability of tPA to promote excitotoxic neuronal death both in vitro and in vivo (Nicole et al., 2001). tPA is recognized as a modulator of glutamatergic neurotransmission (Samson et al., 2006). In vitro, tPA increases NMDA-receptor-mediated calcium influx by interacting with, and then cleaving, the NR1 subunit within its N-terminal domain, and in vivo, blocking the tPA-NR1 interaction prevents permanent cerebral ischemia and reduces the severity of excitotoxic neuronal death in mouse brains (Lopez-Atalaya et al., 2008; Centonze et al., 2003; Benchenane et al., 2007; Kvajo et al., 2004). MMP-9 has emerged as a physiological regulator of NMDA receptor-dependent synaptic plasticity and memory and increases surface trafficking of the NR1-NMDA receptor through integrin β1 signaling (Michaluk et al., 2009). Taken together, these findings suggest a molecular link between tPA →MMP-9→NR1 and targeting this pathway may hold a great promise for blocking tPA's neurotoxicity.

Concluding Remarks

Cerebral I/R injury induces dynamic changes in the BBB permeability, but the underlying mechanisms have remained largely unknown. More efforts are needed to better understand the molecular mechanism underlying the degradation and redistribution of various protein components of the BBB according to the type, severity and duration of cerebral ischemic insults. Since clinical trials indicates that a singular focus on saving neurons alone does not work for stroke, in recent years the concept of the neurovascular unit has emerged as a new paradigm for stroke investigation and therapy. More effective stroke treatment should rescue the integrity of interactions among all cell types within the entire neurovascular unit.

Abundant evidence indicates that MMPs contribute to the BBB disruption during the early phase of stroke. The major challenge with therapeutic interventions of MMPs remains how to accomplish temporal and spatial control of their activity in the brain. In experimental stroke, the therapeutic window for MMP-9 inhibition seems to be narrower than previously estimated and may not extend beyond 24 hours, mainly because a delayed inhibition of MMP-9 exacerbates stroke pathology. Blocking MMPs at a badly chosen time and in nontarget cell types may result in unwanted side effects.

Emerging evidence strongly suggests that MMPs (mainly MMP-9) play a central role in tPA-mediated neurotoxicity in thrombolytic therapy for acute stroke. Thus, pharmacological inhibition of MMPs (at an appropriate time) may hold promise as a safe and effective adjunct therapy for tPA in acute ischemic stroke. In addition to MMPs, several other molecular pathways also have been implicated in tPA-induced neurotoxicity. As discussed above, tPA neurotoxicity is blocked either by co-treatment with neuroserpin or APC or by inhibiting LRP, PDGF-CC, or NMDA receptors during ischemic stroke. Targeting these pathways might offer new combinational therapies for decreasing tPA neurotoxicity and improving stroke therapy. More efforts are needed to characterize both beneficial or detrimental effects of these pathways at different stages of stroke and to establish the time window for tPA co-treatment of acute ischemic stroke.

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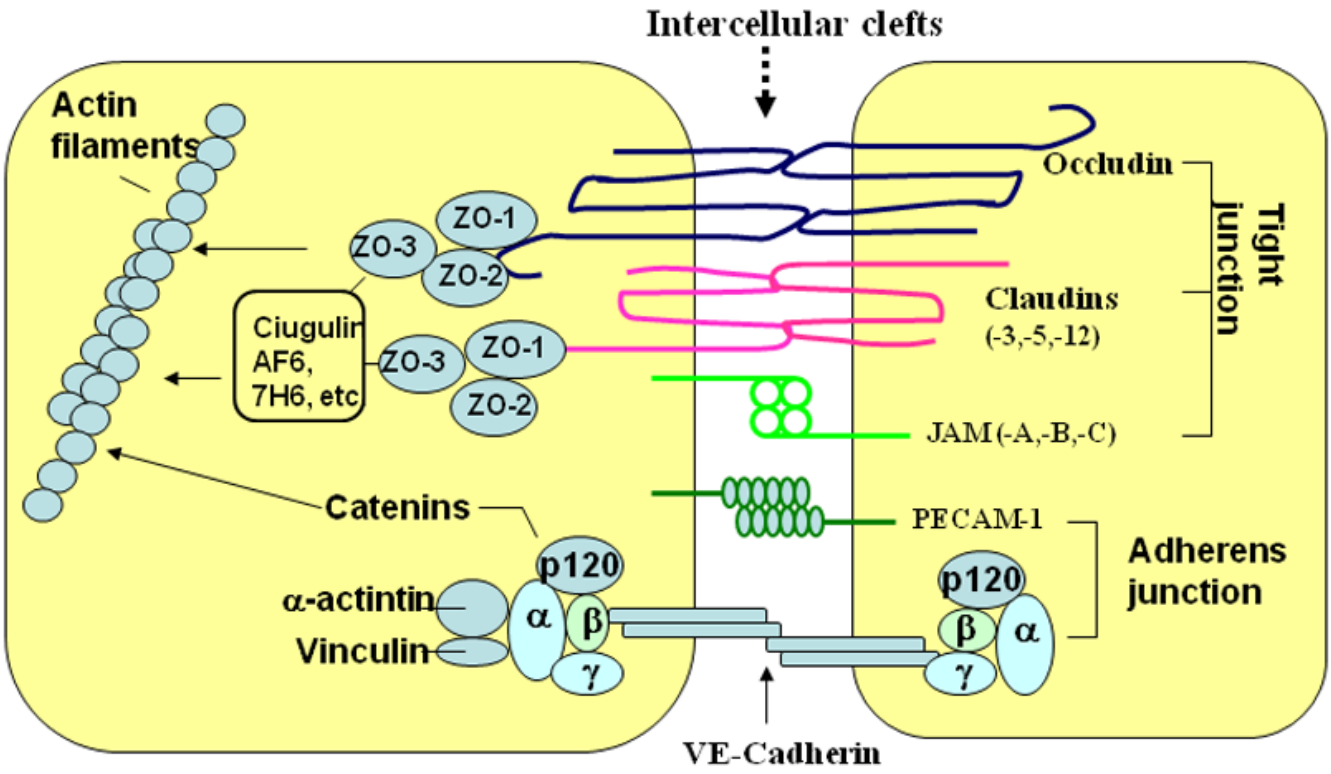

Figure 1.

Schematic diagram of the BBB structure that comprises the tight and adherens junctions. The tight junctions consist of occludin, claudins, and junctional adhesion molecules (JAM). The tight junctions also consist of several accessory proteins necessary to form structural support, including the zonula occluden (ZO) proteins, AF6, 7H6, cingulin, and others. Most of the tight junction components (ZO proteins, claudin, and occludin) have the ability to bind to actin cytoskeleton in brain endothelial cells. The adherens junctions consist of vascular endothelial cadherin (VE-cadherin) and catenin proteins and provide structural integrity and attachment between the cells, and are necessary for formation of tight junctions. Updated from Abbott NJ, 2009 and other sources.

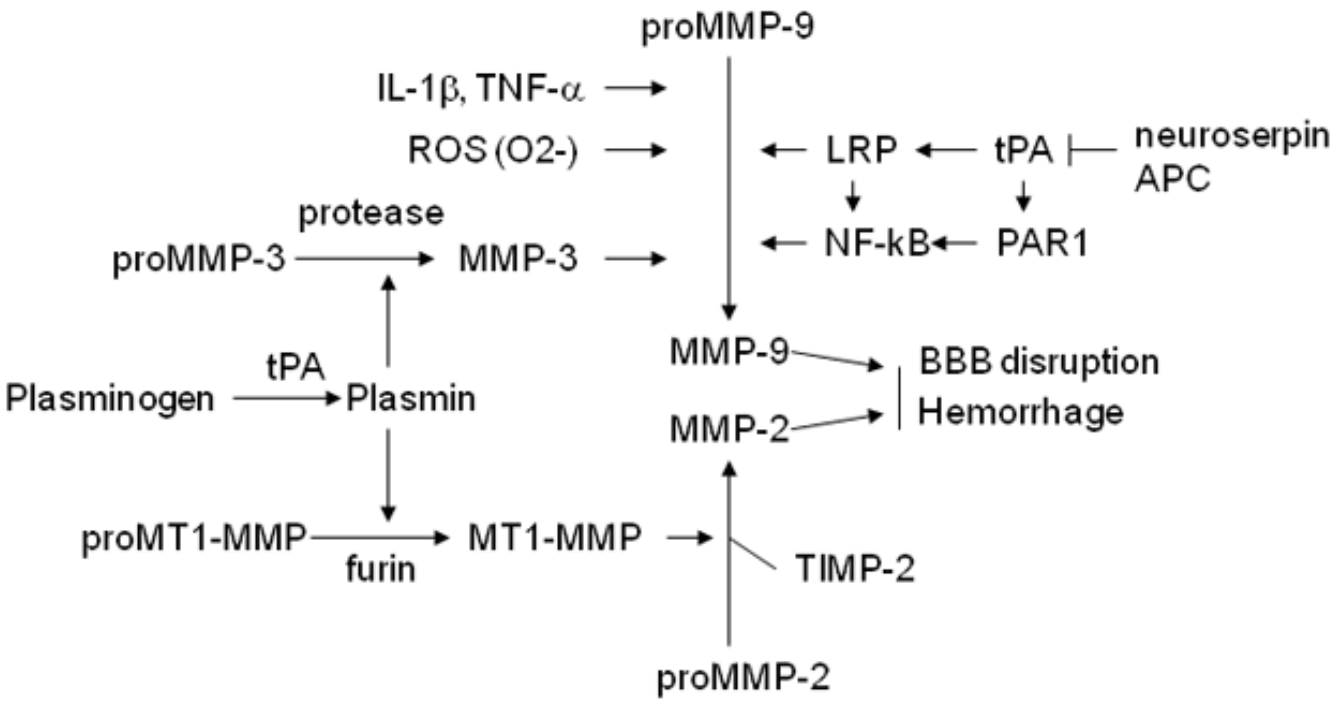

Figure 2.

Mechanisms of activation of MMPs. ProMMP-2 can be activated by membrane-type-1 MMP (MT1-MMP/MMP-14), and the latter can be activated by furin. ProMMP-9 can be activated by MMP-3, tissue type plasminogen activator (tPA), proinflammatory factors (e.g. IL-1β and TNF-α) and reactive oxygen species (ROS). Plasmin can activate both MT1-MMP and MMP-3. tPA can activate both MMP-3 and MMP-9 through multiple pathways. tPA activity can be inhibited by neuroserpin and activated protein C (APC).

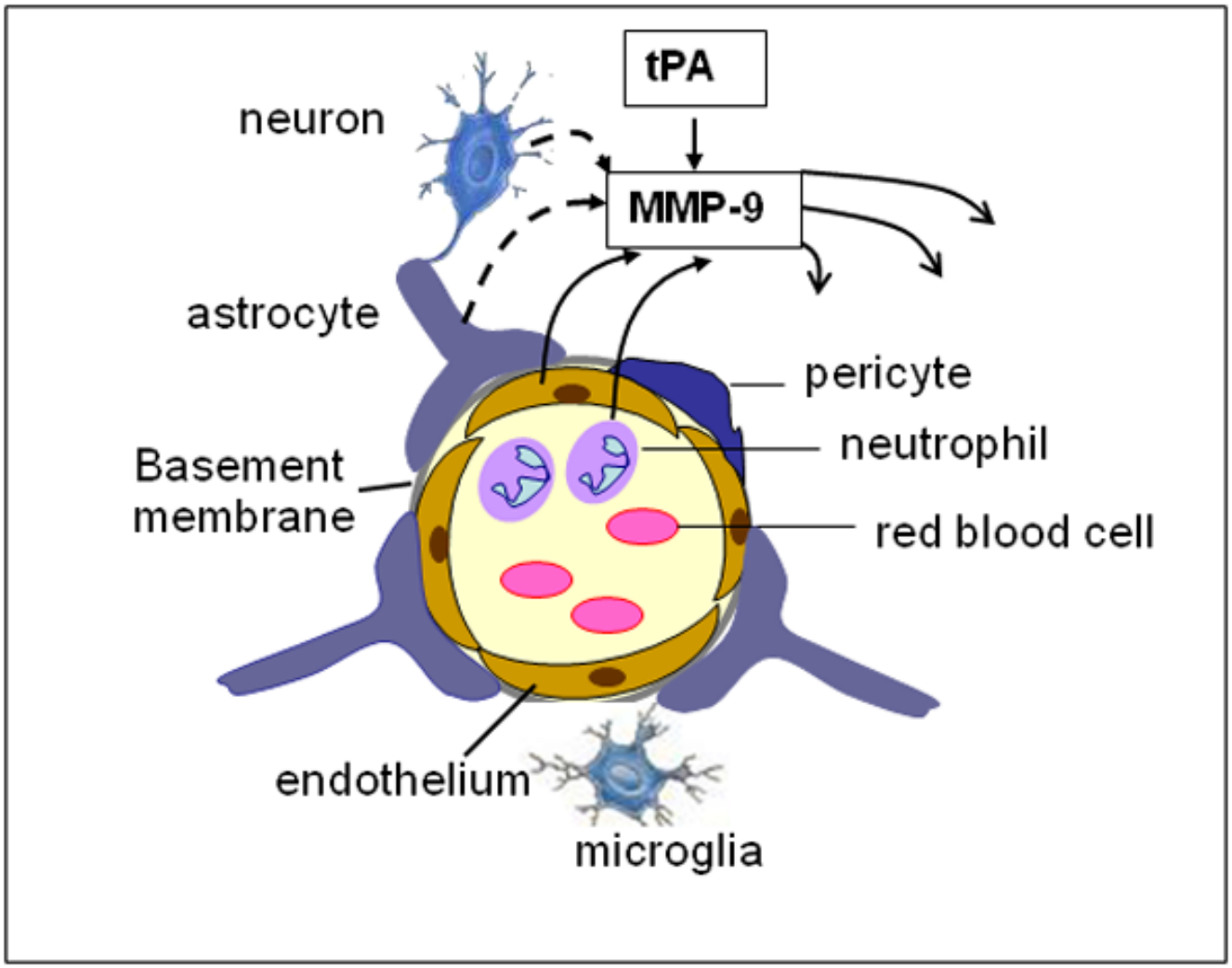

Figure 3.
Schematic diagram of the neurovascular unit that comprises neurons, microvessels (endothelium), astrocytes, and pericytes that reside within the basement membrane. Neurons and microvessels communicate through astrocytes (updated from del Zoppo GJ, 2006 and other sources). There is spatiotemporal change of MMP-9 expression within the neurovascular unit after ischemic stroke. In the acute phase (within 24h), MMP-9 is mainly derived from brain endothelial cells and infiltrating leukocytes (especially neutrophils). In the late phase, MMP-9 is mainly secreted by astrocytes and neurons. Updated from Zlokovic BV, 2006 and other sources.