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Hemorrhagic Transformation After Tissue Plasminogen Activator Reperfusion Therapy for Ischemic Stroke: Mechanisms, Models, and Biomarkers

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Summary

Intracerebral hemorrhagic transformation (HT) is well recognized as a common cause of hemorrhage in patients with ischemic stroke. HT after acute ischemic stroke contributes to early mortality and adversely affects functional recovery. The risk of HT is especially high when patients receive thrombolytic reperfusion therapy with tissue plasminogen activator, the only available treatment for ischemic stroke. Although many important publications address preclinical models of ischemic stroke, there are no current recommendations regarding the conduct of research aimed at understanding the mechanisms and prediction of HT. In this review, we discuss the underlying mechanisms for HT after ischemic stroke, provide an overview of the models commonly used for the study of HT, and discuss biomarkers that might be used for early detection of this challenging clinical problem.

Keywords

blood–brain barrier; hemorrhagic transformation; ischemic stroke; tissue plasminogen activator

Introduction

Worldwide, stroke is among the leading causes of death and severe disability [1–4]. During an ischemic stroke, a blood clot lodges in a small vessel of the brain. The disruption in blood supply leads to rapid loss of brain function and formation of a large, complex infarct region by excitotoxicity, oxidative stress, apoptosis, necrosis, and neurovascular matrix proteolysis. These pathways may occur in conjunction with a common neuroinflammatory response, which perturbs homeostasis within the so-called neurovascular unit [2]. The consequent

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Conflict of Interest

The authors have no conflict of interest.

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breakdown of the blood–brain barrier (BBB) after vessel reperfusion leads to hemorrhagic transformation (HT). BBB breakdown can occur in the absence of any intervention [3–5], but treatment with tissue plasminogen activator (tPA) or surgery can aggravate BBB disruption and increase the risk of HT [1].

tPA is the only drug approved by the United States Food and Drug Administration to treat ischemic stroke. It is a serine protease that catalyzes the conversion of plasminogen to plasmin, which then dissolves the blood clot that produced the stroke. To be effective, tPA must be administered intravenously within the first 3–4 h of the event, owing to risk of HT after ischemic stroke [6]. Unfortunately, only a small percentage of patients with ischemic stroke benefit from thrombolytic therapy. Therefore, it is necessary to find new therapeutic targets that can increase the therapeutic time window of tPA, protect the neurovascular unit from ischemic damage, and prevent development of HT.

Several in vitro and in vivo models of stroke are available that can be used to examine the underlying mechanisms of ischemic stroke and test potential therapeutic interventions. An increased emphasis has emerged on neurovascular mechanisms and in vitro and in vivo models that may ultimately reveal novel combination therapies. Different diagnostic methods have also been explored as ways to help clinicians predict and manage HT. In this review, we briefly discuss the underlying mechanisms of HT in the ischemic brain, provide an overview of ischemic stroke models commonly used to study HT, and describe advances in the early detection of HT.

Mechanisms of HT

Fundamentally, post-stroke HT occurs when BBB permeability increases. A few seconds to a few minutes after ischemia onset, ATP decreases, causing a subsequent loss of Na^+/K^+ ATPase activity. Further rupture of BBB damages the whole neurovascular unit, which consists of the extracellular matrix, endothelial cells, astrocytes, neurons, and pericytes [7]. Thus, neurovascular injury can significantly extend parenchymal damage into irreversible infarction and pan-necrosis [8]. Because the underlying pathways of HT are complex and diverse, we will discuss the mechanisms mainly as they relate to proteolysis, oxidative stress, and leukocyte infiltration.

Proteolysis

Emerging data suggest that HT after tPA therapy for ischemic stroke may be related to dysregulated extracellular proteolysis within the neurovascular matrix [9,10]. Degradation of the basic components of the BBB produces leakage and rupture, which aggravates brain edema and enhances brain damage [11]. Structural evidence shows that the basic mechanism leading to extravasation of blood is disruption of the BBB. Although many proteases are expressed in the brain under normal and ischemic conditions, both animal and human studies have indicated that the matrix metalloproteinase (MMP) family and the tPA system play a central role [12].

Preclinical Studies—MMPs comprise a large family of zinc endopeptidases that are responsible for degrading the matrix substrates in brain [13]. In the past 10 years, studies

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have shown that the expression of MMP-2, MMP-3, and MMP-9 rapidly increases in the ischemic brain, and that the increase in these MMP activities is closely related to infarct extension, neurologic deficits, and HT [14]. Some studies provide direct evidence that targeting MMP-2 effectively prevents the loss of collagen and occludin and protects against HT after ischemia and reperfusion [15]. Studies also have shown that MMP-2 levels are highly related to the degradation of tight junction (TJ) proteins, basal lamina, and neuronal injury after ischemia [16]. Of the MMPs, MMP-9 has been the most extensively studied in acute ischemic stroke. MMP-9 activity is significantly elevated in human brain tissue and serum after ischemic stroke, and a high level of serum MMP-9 independently forecasts HT in patients treated with tPA [17]. MMP-2, on the other hand, plays a key role in the initial opening of the BBB after cerebral ischemia [18]. According to another report, MMP-9 mRNA concentration was almost three times higher in patients who died than in those who survived after acute stroke [19]. Thus, increased MMP-9 mRNA may predict poor outcome and mortality after stroke. Recently, Demir et al. [20] reported that plasma MMP-9 level increased significantly in the acute phase of ischemic stroke and correlated with disease severity and infarct volume of patients. These results are in line with studies showing that MMP-9-positive neutrophil infiltration is associated with enhanced erythrocyte extravasation and severe degradation of collagen IV in the basal lamina after human ischemic stroke [12]. MMP-9, however, is only associated with HT and severe edema [21]. Elevated serum pro-MMP-10, which is associated with tumor necrosis factor (TNF)-α, is a new marker of brain injury and poor outcome after acute ischemic stroke [22].

In animal models, BBB disruption seems to derive from the pleiotropic protease actions of tPA, which include activation of apoptosis [23], cleavage of the N-methyl-D-aspartate (NMDA) NR1 subunit [24], and activation of other extracellular proteases, such as MMP-9 [25]. MMP-9 has been shown to reduce TJ proteins (claudin-5, occludin, ZO-1) in cultured brain endothelial cells and in an animal model of cerebral ischemia [26]. Experimental studies suggest that MMP-9 is important in the delayed opening of the BBB after ischemic stroke. In the rat model of transient middle cerebral artery occlusion (MCAO), Planas et al. [27]showed that MMP-9 is induced and activated from 4 h to 4 days. In a mouse model of MCAO, Kaplan et al. [28] showed that MMP-9 expression and activity were elevated and BBB permeability increased as early as 2–4 h after ischemia. tPA is widely expressed in the developing and mature brain [29] and by neurons and microglia [30]; it is thought to mediate neuronal death and microglial activation after excitotoxic injury [31]. In addition to its impact on the intravascular compartment, exogenous tPA can cross the intact or injured BBB into the brain parenchyma, where it produces neurotoxic effects [32]. In a cell culture system, tPA interacts with the NR1 subunit and thereby reduces calcium current amplification during ischemic excitotoxicity [24]. In addition, tPA amplifies excitotoxic neuronal death in hippocampus by degrading interneuronal laminin and disrupting prosurvival cell–matrix signaling [33]. Evidence shows that tPA can upregulate and activate various MMP family members (especially MMP-3 and MMP-9) [12]. Indeed, the “tPA-induced MMP-9” hypothesis is generally accepted and supported by experimental evidence [10]. In vivo experiments have shown that tPA thrombolytic therapy after experimental embolic stroke increases brain levels of MMP-9 and that tPA combined with MMP inhibitors can reduce HT and brain damage [1]. Because angiogenic factors have a potential

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role in MMP-9 activation [34] and vascular permeability [35,11], their role in HT after ischemic stroke also deserves further investigation.

Clinical Studies—Available evidence indicates that specific inhibition of MMP activity after the onset of stroke or immediately after stroke improves neurologic outcomes [36,37]. A recent clinical trial showed that minocycline can reduce plasma MMP-9 level and improve neurologic outcomes in patients with acute ischemic stroke who are treated with tPA [38]. Cui et al. [39] recently used an embolic cerebral ischemia mouse model to assess the effects of (4-phenoxyphenylsulfonyl) methylthiirane (known as SB-3CT), the first mechanism-based inhibitor of MMP selective for gelatinases. They showed that SB-3CT could antagonize neuronal apoptosis after transient focal cerebral ischemia. Methods of MMP-9 inhibition include lentiviral-mediated MMP-9 gene silencing [40], delivery of recombinant TIMP-1 in its native form [41], and use of PLGA (poly lactic-co-glycolic acid) nanoparticles [42]. One study suggested that a liposomal formulation of siRNA might be used in vivo to silence the MMP-9 gene and could be used as a treatment option for patients with cerebral ischemia [43]. Therefore, the available literature indicates that inhibition of MMP-9 could be a therapeutic strategy for treatment of ischemic stroke. As some have suggested that HT after tPA use may stem from activation of MMP-9, the combination of MMP-9 inhibition with tPA, might be particularly beneficial [17].

Oxidative Stress

Preclinical Studies—It is becoming increasingly clear that oxidative stress plays an important role in BBB dysregulation during HT in the ischemic brain. Two interesting studies that used the cerebral ischemia-reperfusion model showed that oxidative stress mediates BBB damage by MMP activation in mice lacking copper/zinc-superoxide dismutase [38] and that treatment with the free radical scavenger a-phenyl-tert-butyl nitrone significantly reduces tPA-induced HT in embolic focal ischemia [44]. In fact, the generation of free radicals and oxidative damage in the BBB is recognized as the main trigger mechanism of HT after transient focal cerebral ischemia [45].

In the central nervous system, intense and prolonged oxidative stress causes the overexpression and release of proinflammatory cytokines, which regulate the expression of MMPs and TIMPs [46]. According to one report, Aβ1–40 perivascular deposition reduced the expression of TJ proteins claudin-1 and claudin-5 and increased the expression of MMP-2 and MMP-9 [47]. In cultured endothelial cells, Aβ1–42 enhanced permeability by disrupting expression of ZO-1 in the plasma membrane and increasing the secretion of intracellular calcium and MMPs [48].

Clinical Studies—The molecular mechanisms of oxidative-induced BBB damage include: 1) anoxia; 2) inflammation, proinflammatory cytokines, and chemokines; and 3) β-amyloid peptides and cerebral amyloid angiopathy [48]. Anoxia is known to change BBB permeability and TJ protein expression in cerebral capillaries [49]. Cytokines such as interleukin (IL)-1, IL-6, and TNF-α are increased in plasma and cerebrospinal fluid of acute ischemic stroke patients and seem to increase the risk of progression or recurrence [50]. In particular, TNF-α, which stimulates MMP expression and synthesis, is considered to be one

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of the most important proinflammatory cytokines and an important link between the proinflammatory cytokine network and the local increase in MMP proteolytic activity [46]. Chemokines CCL-2 and CXCL-8 also have been reported to be responsible for increased BBB permeability, as CCL-2 is produced by both astrocytes and endothelial cells in the late stages of hypoxia/reoxygenation-induced BBB damage [51]. Finally, β-amyloid peptides affect small blood vessels in the brain by inducing cerebral amyloid angiopathy [52].

Leukocyte Infiltration

Preclinical Studies—Leukocyte recruitment, activation, and infiltration may play a pivotal role in HT. With the interaction of inflammatory leukocytes and cerebral endothelial cells, adhesion molecules such as V-CAM and/or I-CAM might contribute to endothelial dysfunction and cell damage [53]. Inflammatory responses become apparent when leukocytes infiltrate the ischemic brain and begin to express and/or activate other proteins such as selectins, cytokines, integrins, and MMPs [54]. In an embolic stroke model in rats, early administration of proteasome inhibitor PS-519 or minocycline reduced inflammation and MMP-9 levels, respectively, and thereby extended the effective time-window for tPA to 6 h without increasing the incidence of HT [55]. Interestingly, one study showed that neutrophil depletion has no effect on gelatinase activity or HT in a suture MCAO model in rats [56].

Clinical Studies—Leukocyte infiltration plays an important role in triggering BBB disruption and HT. Leukocytes are critical to the neuroinflammatory response. Various leukocyte chemoattractants released in the ischemic zone attract leukocytes across the BBB into the brain [2]. Xing et al. [57] reported that white blood cell count was significantly higher in patients that experienced HT than in those that did not. The subsequent enhanced leukocyte infiltration might damage microvascular endothelial cells, mediate the opening of the BBB, and lead to HT.

Microglia and Astrocytes

Microglia, tissue-resident macrophages, and astrocytes are components of the BBB [58]. However, few studies have examined the role of microglia and astrocytes in HT. A recent in vitro study showed that ischemic neurons are able to activate astrocytes to produce vascular endothelial growth factor and induce endothelial barrier disruption. Since the latter is closely associated with HT, it is likely that astrocytes also play a role in HT [59]. Interestingly, although both astrocytes and microglia express MMP-9 in ischemic brain tissue [60], del Zoppo et al. [61] reported that it is the microglia–astrocyte interaction, not astrocytes alone, that generates MMP-9 and contributes to ischemia-induced HT and edema. Future studies may need to determine whether the microglia–astrocyte interaction affects microglial phenotype, thereby affecting HT after ischemic stroke.

Models for the study of HT

Although several in vitro and in vivo models are available that can be used to study the underlying mechanisms of ischemic stroke, no in vitro model has been established to closely mimic HT. In this section, we will discuss the animal models that are most commonly used

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for the research of HT in ischemic stroke. However, the exact protocols are beyond the scope of this review and have been described in detail by others [62].

Preclinical Animal Models

Animal models can be used to examine the complex pathophysiology of HT and identify effective prevention and treatment methods [63]. Rodents are most commonly used because their brains and physiology are similar to those of humans. Additionally, because they are small in size, they are easy to handle and relatively inexpensive.

Researchers most frequently use experimental focal cerebral ischemia as a model to study HT because it closely mimics pathophysiologic changes of HT during and after the occurrence of ischemic stroke in humans [62]. Some of the protocols for inducing local cerebral ischemia and HT are described below.

Mechanical Model of MCAO—This ischemic stroke model is purely mechanical and does not use thrombolytic drugs such as tPA. Proximal MCAO (large vessel occlusion) is among the most commonly used procedures in HT research. Proximal MCAO is usually produced by direct mechanical obstruction, often by inserting a silicone-coated nylon filament into the carotid artery and then advancing it to the circle of Willis to block the middle cerebral artery origin. The severity of HT after ischemic injury can be altered by varying the duration that the filament remains in place. If the filament is removed to allow reperfusion, it is considered transient MCAO. In a related model, the filament can be left in place permanently, with no reperfusion. Permanent MCAO causes larger infarct volumes than does transient MCAO [64]. Short-term proximal MCAO induces expression of heat shock proteins and causes apoptosis of selective neurons in ipsilateral striatum [65] and brain cortex [66]. Prolonged obstruction can cause cerebral infarction that includes the cortex and striatum and is associated with HT and higher mortality [67]. Compared with other methods described in this article, proximal MCAO requires no craniotomy [68], is highly reproducible, and produces focal brain damage and HT that are similar to those in human stroke [69]. The advantage of this mechanical model is that thrombolytic drugs such as tPA are not required to induce HT. Additionally, increasing the duration of MCAO increases the rate and severity of HT.

Thrombolysis model of HT—In the thrombolysis model, blood clot formation is produced first in the target vessel, and then thrombolytic drugs are used to dissolve the clot, simulating the clinical process of HT in ischemic stroke. This model is of great interest because it is similar to human ischemic stroke and because it responds to thrombolytic therapy.

In the typical protocol for thromboembolic vascular occlusion, autologous blood clots are injected directly into the internal carotid artery [70]. Alternatively, purified thrombin can be injected directly into the middle cerebral artery to cause reproducible ischemic injury volumes. Then, thrombolytic and anticoagulant medications are used to induce HT in this model. These drugs include warfarin, aspirin, and tPA. Although long-term oral anticoagulation therapy with warfarin is widely used to prevent cardioembolic ischemic stroke, preclinical studies have shown that warfarin can aggravate HT after cerebral

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ischemia [71,72]. However, certain drugs, such as SMTP-7 [73] or cilostazol [74] are able to reduce warfarin-induced HT after cerebral ischemia in mice. Aspirin therapy is often used in patients with cardioembolic stroke and is associated with few reports of HT [75,76]. The most commonly used thrombolytic drug for induction of HT in animal models is tPA. Garcia-Yebenes et al. [77] have verified a reproducible mouse model of HT in which administration of tPA is delayed after in situ thromboembolic stroke. In this model, delivery of recombinant tPA (rtPA) at 3 h after stroke led to a significant reduction in brain damage. However, another study showed that delayed administration of rtPA at 6 h after stroke caused rapid deterioration of ischemia-induced microvascular barrier damage, thus increasing HT [78].

Animal studies have provided most of our knowledge regarding the mechanism of HT in focal cerebral ischemia. Because there are many forms of human stroke, no single animal model can exemplify all of the variables that affect HT in humans. Moreover, the lack of a model that fully simulates human stroke and HT is one of the main reasons for the failure of many drugs to translate into effective stroke therapy in clinical trials. Therefore, we still need to establish better and more clinically relevant animal models to mimic human HT.

Biomarkers of HT

Clinical Risk of HT

In the past two decades, various treatment strategies for acute ischemic stroke have become available. Although these treatments have potential benefit, they also have their own inherent risks. The strong fibrinolytic activity of rtPA may increase the risk of HT. Moreover, endovascular treatment methods may cause mechanical damage to the vascular endothelium and increase the risk of hemorrhage. The NINDS rtPA Stroke Study has shown low rates of both asymptomatic (4.5%) and symptomatic (6.4%) HT (4.5%) [79]. The Safe Implementation of Thrombolysis in Stroke-Monitoring Study (SITS-MOST), a large intravenous rtPA observational study, also showed a relatively low rate of asymptomatic hemorrhage (9.6%), but an additional 7.3% of patients had symptomatic hemorrhage [80]. On the other hand, the ECASS-II and -III trials have shown higher rates of asymptomatic hemorrhage, with 39.6% and 27%, respectively [81]. Compared to the rates with intravenous fibrinolysis, intraarterial drug administration seems to cause higher rates of symptomatic and asymptomatic hemorrhage [82].

Recently, intravenous and intraarterial methods were combined in some specialized stroke centers. In these trials, patients received intravenous fibrinolysis in transport to the endovascular suite for further intraarterial intervention. The Emergency Management of Stroke (EMS) Bridging trial [83] and the Interventional Management of Stroke (IMS)-I, -II, and -III trials [84] have assessed this combined approach. The rates of asymptomatic hemorrhage in the EMS, IMS-I, IMS-II, and IMS-III trials were 11.8%, 43%, 32.1%, and 27.4%, respectively, while rates of symptomatic HT were 23.5%, 6.3%, 9.9%, and 6.2%, respectively, showing that combination therapy is at least as safe as the special method of intraarteral administration. However, it is important to emphasize that we must be careful when comparing different clinical trials with distinct eligibility criteria and study designs.

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Radiographic and Magnetic Resonance Imaging

Considering that thrombolytic therapy is used for acute ischemic stroke, it would be helpful to be able to predict possible HT development [85]. It has been reported that the first neuroimaging parameters on pretreatment computed tomography (CT) scans can predict HT after thrombolytic therapy [86]. Using dynamic perfusion CT, Lin et al. [87] reported that in 88% of the patients with ischemic stroke, microvascular permeability increased within the first 3 h after stroke onset. Neeb et al. [88] successfully adapted the European Cooperative Acute Stroke Study (ECASS) CT classification of HT for usage in magnetic resonance imaging (MRI). In their study, classification of HT had nearly perfect inter-observer agreement. Thus, use of CT or MRI for HT prediction can be used for safety evaluation of clinical trials.

Recent studies have shown that cerebral microbleeds (CMBs) found on T2-weighted MRI may predict symptomatic cerebral hemorrhage [89]. MRI with T2-weighted gradient echo sequence has a high sensitivity for iron-containing compounds and is useful for detecting CMBs that contain hemosiderin. The relationship between CMBs on T2-weighted images and HT after ischemic stroke has been examined through various surveys [90]. The evidence is conflicting regarding whether CMBs are significantly associated with HT [91,92]. Moriya et al. [92] found that the volume of ischemic tissue in terms of ASPECTS-DWI score (the Alberta Stroke Program Early Computed Tomography Score-diffusion-weighted imaging) seems to be a useful marker for predicting HT, whereas CMBs on T2-weighted images may not predict HT in patients who receive intravenous rtPA treatment. In patients who do receive rtPA, high signal intensity volume on DWI is an independent predictor of symptomatic cerebral hemorrhage [85]. Kimura et al. [93] used T2-weighted MRI to investigate the frequency and clinical factors of new-extraischemic microbleeds (new-EMBs) after tPA injection. They found that new-EMBs occurred quickly after tPA injection in 4.9% of patients and that patients with new-EMBs are likely to have symptomatic extraischemic hemorrhage. Permeability images derived from magnetic resonance perfusion images are sensitive to BBB disorder and have been shown to correlate with subsequent development of HT in acute ischemic stroke [94]. Although certain CT or MRI techniques may predict HT in humans, no imaging techniques are available to reliably predict HT in animal models.

Blood Biomarkers for HT

It is important to identify early biomarkers of HT so that we can find new treatments and predict clinical outcomes of patients. Ideal molecular markers would be those that can be measured at the bedside in biological fluids such as blood or urine. One potential candidate is MMP-9 in blood. Hyperacute MMP-9 plasma level seems to be a powerful predictor of HT after tPA thrombolysis [95]. Serum level of calcium binding protein (S100B), an astroglial cytoplasmic protein, also has been shown to be associated with HT after ischemic stroke. A high level of S100B before thrombolytic therapy predicts further parenchymal hemorrhage and is an independent risk factor for HT [96]. Endogenous hemostatic state may also predict the risk of bleeding, particularly after thrombolytic therapy. Two studies have tried to evaluate blood levels of fibrinolysis inhibitors, including plasminogen activator inhibitor type-1 (PAI-1) and thrombin-activated fibrinolysis inhibitor (TAFI), before

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thrombolysis. One study found that baseline levels of PAI-1 and TAFI predicted further symptomatic intracranial hemorrhage [97], but the other failed to detect a difference between patients in levels of PAI-1, TAFI, or other hemostatic markers [98]. More investigations may be required to validate the utility of these potential markers in the clinic. Recently, plasma levels of F2-isoprostanes (free radical-induced neuronal arachidonic acid peroxidation) were found to be higher in patients with acute ischemic stroke than in controls [99] and were correlated with plasma MMP-9 level [38]. Whether F2-isoprostanes could be used as a blood biomarker for HT needs to be determined.

Conclusion and perspective

One of the difficult clinical issues we face in the stroke field is reducing the risk of HT after rtPA administration. Recent preclinical and clinical studies have provided important information about the cellular and molecular mechanisms and biomarkers of HT after ischemic stroke. Using laboratory and CT or MRI techniques in carefully selected stroke patients, it is now feasible to translate preclinical approaches into clinical trials. A better understanding of the cellular and molecular mechanisms of HT and the ability to identify reliable predictive biomarkers of HT will have profound clinical implications for patient management. Additionally, combined strategies that target multiple molecules and different signaling pathways may offer great promise for the prevention and/or treatment of HT after stroke.

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