Therapeutic Strategies to Attenuate Hemorrhagic Transformation After Tissue Plasminogen Activator Treatment for Acute Ischemic Stroke

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This review focuses on the mechanisms and emerging concepts of stroke and therapeutic strategies for attenuating hemorrhagic transformation (HT) after tissue plasminogen activator (tPA) treatment for acute ischemic stroke (AIS). The therapeutic time window for tPA treatment has been extended. However, the patients who are eligible for tPA treatment are still <5% of all patients with AIS. The risk of serious or fatal symptomatic hemorrhage increases with delayed initiation of treatment. HT is thought to be caused by 1) ischemia/reperfusion injury; 2) the toxicity of tPA itself; 3) inflammation; and/or 4) remodeling factor-mediated effects. Modulation of these pathophysiologies is the basis of direct therapeutic strategies to attenuate HT after tPA treatment. Several studies have revealed that matrix metalloproteinases and free radicals are potential therapeutic targets. In addition, we have demonstrated that the inhibition of the vascular endothelial growth factor-signaling pathway and supplemental treatment with a recombinant angiopoietin-1 protein might be a promising therapeutic strategy for attenuating HT after tPA treatment through vascular protection. Moreover, single-target therapies could be insufficient for attenuating HT after tPA treatment and improving the therapeutic outcome of patients with AIS. We recently identified progranulin, which is a growth factor and a novel target molecule with multiple therapeutic effects. Progranulin might be a therapeutic target that protects the brain through suppression of vascular remodeling (vascular protection), neuroinflammation, and/or neuronal death (neuroprotection). Clinical trials which evaluate the effects of anti-VEGF drugs or PGRN-based treatment with tPA will be might worthwhile.

Key words: tPA, Therapeutic time window, Hemorrhagic transformation, Vascular protection, Brain protection

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Introduction

Tissue plasminogen activator (tPA) is the only thrombolytic drug approved to treat acute ischemic stroke (AIS) and is a class-I recommendation in the American Heart Association/American Stroke Association guidelines. The guidelines for the administration of tPA were revised to extend the therapeutic time window (within 4.5 h after the onset of symptoms) in 2012. However, the patients who are eligible for tPA

treatment are still between 3.4% and 5.2% of all patients with AIS because of the very narrow therapeutic time window¹⁾. In the pooled analysis, the risks of serious or fatal symptomatic hemorrhage increased with later initiation of treatment²⁾. The third International Stroke Trial sought to determine whether a wider range of patients undergoing tPA treatment up to 6 h from stroke onset would benefit³⁾. At 6 months, the patients in the tPA group scored better on the Oxford handicap scale than those in the control group. However, fatal or nonfatal symptomatic intracranial hemorrhages occurred within 7 days in 7% of the patients in the tPA group versus 1% of the patients in the control group. Additionally, a very recent study has demonstrated that early treatment is very important for patients with severe strokes because of the increasing risk of symptomatic hemorrhagic transfor-

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mation (HT), even within 4.5 h of onset⁴). Therefore, attenuation of the incidence of HTs after tPA treatment is an important therapeutic strategy against AIS, and it will enable extension of the therapeutic time window and increase the number of patients who are eligible for tPA treatment and the probability of achieving excellent outcomes.

A retrospective clinical study has shown that early disruption of the blood-brain barrier (BBB) after tPA administration, which was indicated by early gadolinium enhancement, predicted a higher risk for symptomatic HT⁵). Moreover, experimental animal models have revealed that the incorrect timing of reperfusion by thrombolysis increases the incidence of intracerebral HT⁶). tPA itself is neurotoxic, and it aggravates the neurodamage caused by glutamic acid release after ischemia if it leaks into the brain parenchyma. In addition, tPA promotes the infiltration of leukocytes and activated microglia and the production of free radicals in ischemic lesions⁷). Furthermore, vascular remodeling factors are upregulated, and microvascular structures are destabilized after cerebral ischemia. These factors also play roles in BBB disruption. Thus, these observations suggest that these factors play dual roles, simultaneously harmful and beneficial, and have diverse heterogeneity, which results in a biphasic clinical course⁸). Multiple cells, such as neurons, astrocytes, microglia, pericytes, and endothelial cells, make up the neurovascular unit (NVU) and are involved in neurovascular dysfunction in the acute phase of ischemic stroke⁹). Alterations in the remodeling factors in multiple target cells might be a therapeutic strategy for attenuating HT after tPA treatment in patients with AIS. Eventually, single-target therapies might be insufficient for attenuating HT after tPA treatment in patients with AIS.

In this Review, we describe the mechanisms underlying HT that occurs after tPA treatment in patients with AIS. In addition, we briefly outline the therapeutic vascular and brain protective strategies for attenuating HT after tPA treatment and improving the therapeutic outcomes of patients with AIS.

Mechanisms of Intracerebral HT After tPA Treatment

In order to suppress tPA-induced HT by BBB disruption, an understanding of the underlying mechanisms is essential. The major causes of disruption of the BBB, which is involved in the intracerebral HTs that occur after tPA treatment, are the following: (1) cerebral ischemia/reperfusion injury, (2) the direct toxicity of tPA, (3) inflammation, and (4) remodeling factor-mediated effects. It is important to understand

the pathophysiologies underlying the disruption of the BBB in order to attenuate intracerebral HT after tPA treatment (**Fig. 1**). Modulating these pathophysiologies are direct therapeutic strategies.

Reactive Oxygen Species Produced by Cerebral Ischemia/Reperfusion

Cerebral ischemia/reperfusion results in the activation of several reactive oxygen species (ROS)-generating enzymatic systems. In ischemia, the resulting increase in cytosolic Ca^{2+} activates the superoxide-producing enzyme nicotinamide adenine dinucleotide phosphate-oxidase through protein kinase C and nitric oxide (NO) that are derived from neuronal nitric oxide synthase (NOS)^{10, 11}). The increased ROS that are produced by ischemia-reperfusion can disrupt the NVU through damage to endothelial cells, pericytes, smooth muscle cells, and astrocytes. This increases the likelihood of HT through increased BBB permeability. Damage of the NVU at the capillary level by ROS species might predispose to petechial hemorrhage, whereas ROS injury to both endothelial cells and pericytes at the small arteriolar level could produce larger parenchymal hemorrhages.

A number of experimental models have shown that ROS are involved in early HT. After 2 h of focal cerebral ischemia and 3 h of reperfusion, ROS levels were increased in microvessels and astrocytic endfeet¹²). The ischemia-induced generation of ROS occurs prior to the upregulation of matrix metalloproteinases (MMPs)¹³). Therefore, ROS play important roles in very early HT.

The experimental evidence implicating oxidative stress in stroke suggests the use of a combination treatment of tPA and radical-trapping agents (free radical scavengers). However, the translation of these fundamental concepts into clinical applications has proven challenging. In animal models of AIS, the free radical-trapping agent NXY-059 had shown promise as a neuroprotectant. SAINT I and II, which were randomized, placebo-controlled, double-blind trials, were then conducted to investigate the efficacy of NXY-059 in patients with AIS. However, these trials failed to reduce HT and improve the outcomes of the patients with AIS¹⁴). Edaravone is another free radical scavenger that can reduce HT in rat stroke¹⁵). Notably, the postmarketing registry of the PROTECT4.5 trial on edaravone treatment in acute cerebral infarction in the 4.5-h time window has shown that the frequency of intracerebral HT is lower with the use of edaravone in combination with tPA than with tPA alone¹⁶). In addition, edaravone might be a good partner to use in combination therapy with tPA to enhance recanaliza-

Fig. 1. Mechanisms of intracerebral hemorrhagic transformation after tPA treatment and therapeutic targets

BBB, blood-brain barrier; MMP, matrix metalloproteinase; ROS, reactive oxygen species; tPA, tissue plasminogen activator

tion and reduce HT¹⁷). The results of a tPA and edaravone combination therapy (YAMATO) study are not yet available. However, a worldwide clinical trial to assess the efficacy of edaravone has not been conducted. Therefore, strong evidence for the efficacy of edaravone is needed. Isoflurane may increase ROS by inhibiting superoxide dismutase and catalase. It increases HT in rats with focal ischemia¹⁸). Hydrogen gas, which might reduce oxidative stress in the brain, reduces hyperglycemia-enhanced HT in a rat stroke model¹⁹). However, small animal studies cannot delineate the evidence for suppressing HT and are less likely to translate to the clinic.

Direct Endothelial Injuries by tPA

tPA is thought to cause neuronal damage and be directly involved in BBB disruption (**Fig. 2**). The results of animal studies have indicated that tPA increases neuronal damage after focal cerebral ischemia that is mediated by glutamatergic receptors by modifying the properties of the N-methyl-D-aspartate (NMDA) receptor²⁰). In addition, tPA potentiates apoptosis in ischemic endothelium by shifting the apoptotic pathways from caspase-9 to caspase-8, which directly activates caspase-3²¹). Activated protein C,

which is a serine protease with anticoagulant activity, inhibits tPA-induced caspase-8 induction and caspase-3 activation in endothelium and hemorrhage^{21, 22}). tPA cleaves the low-density lipoprotein receptor-related protein (LRP) in the plasma membrane of astrocytes, which are located around blood vessels, and the cleaved extracellular fragments induce MMP-9 through nuclear factor- κ B pathway activation²³). In addition, tPA promotes neutrophil degranulation and MMP-9 release²⁴). The administration of tPA results in the degradation of the protein components of the basal lamina and extracellular matrices by plasmin and MMP-9²⁵⁻²⁷) (**Fig. 3**). MMP-9 might directly degrade tight junction proteins^{27, 28}). Several mechanisms of tPA-induced BBB disruption have been described. However, no evidence currently exists for direct injury effects of tPA in the degradation of tight junction proteins of the BBB in the acute time frame of the use of tPA because the administration of tPA within a few hours after onset is not clinical evidence of the induction of HT²⁷).

BBB Disruption by Inflammation

The inflammatory response to ischemic stroke expands cerebral infarct volume and induces BBB dis-

Fig. 2. The cascade of tPA-induced adverse effects

APC, activated protein C; LRP, Low-density lipoprotein receptor-related protein; MMP, matrix metalloproteinase; NMDA, N-methyl-D-aspartate; tPA, tissue plasminogen activator

ruption. Leucocytes infiltrate in increasing numbers within several hours. Macrophages (both microglia-derived and blood-derived) are visible within 24 h, with decreasing numbers of neutrophils seen within 48 h²⁹. Activated microglia and infiltrating inflammatory cells secrete proinflammatory mediators that amplify the inflammatory response, as well as various effector molecules, including proteases, prostaglandins, and ROS, such as NO, through inducible NOS (iNOS), which can directly damage cells or the extracellular matrix³⁰. Cytokines might also directly lead to cell death³¹. Damage to the endothelium and other components of the BBB can lead to uncontrolled vasogenic edema, microvascular ischemia, or HT.

Microglia and neutrophils are sources of MMP-9³². MMP-9 induction also results from tPA/LRP interactions in microglia³³. MMPs directly degrade tight junction proteins, including occludin and claudin-5, and components of the extracellular matrix of the basement membrane, such as fibronectin, laminin, collagen, and proteoglycans, and MMPs thereby produce NVU impairments, leukocyte infiltration, brain edema, and HT^{25, 26, 34} (**Fig. 3**). Again, infiltrated leukocytes might, in fact, aggravate BBB leakage as tPA has been shown to promote the degranulation of neutrophils, which results in the massive release of MMPs²⁴.

In addition, tPA itself might mediate neuroin-

flammatory processes. Briefly, tPA promotes microglial chemotaxis by the processing of monocyte chemoattractant protein-1 (MCP-1)³⁵, which is a chemokine, and activating microglia following excitotoxic injury and expanding inflammation³⁶. Moreover, tPA activates microglia by binding to LRP-1³³ or annexin-II³⁷ (**Fig. 2**). However, microglia are a source of tPA, and tPA deficiency reduces microglia activation by bacterial lipopolysaccharide stimuli, which suggests that tPA acts on microglia activation in an autocrine fashion³⁸. Inflammatory cells are strongly associated with HT through several mechanisms (**Fig. 1**). Thus, the suppression of inflammation might be an important strategy to attenuate HT.

Minocycline and a pan-MMP inhibitor might also be therapeutic candidates. Minocycline, which is a tetracycline derivative, reduces inflammation and MMP-9 activation and protects against focal cerebral ischemia^{39, 40}. Furthermore, minocycline is clinically safe and well tolerated in combination with tPA⁴¹. However, a multinational clinical trial of minocycline has not been conducted. In contrast, the focal ischemic model of experimental stroke proposes that the administration of the monoclonal antibody against the programmed death-1 receptor (PD-L1), which is expressed on T cells, has been reported to reduce infarct volumes and improve neurological outcomes after 96 h of reperfusion⁴². Thus, fingolimod, which

Fig. 3. The schema of protease-mediated BBB disruption

BBB, blood-brain barrier; ECM, extracellular matrix; MMP, matrix metalloproteinase; tPA, tissue plasminogen activator

essentially traps lymphocytes in lymph nodes, attenuates the neurological deficits and reduces infarct volume after *in situ* thromboembolic occlusion of the middle cerebral artery⁴³. The combination of fingolimod and tPA improves the neurological outcomes of the thrombolytic therapy and reduces the risk of HT that is associated with the delayed administration of tPA. These results support the use of the available humanized anti-PD-L1 antibody and fingolimod in the treatment of human stroke subjects. In fact, a clinical trial of fingolimod has started.

The Remodeling Factor Mechanisms of HT

The classical definition of ischemic penumbra is the region of salvage of peri-infarct lesions by any treatment⁴⁴. Thus, one of the definitions of an ischemic penumbra is a region consisting of multiple molecules⁴⁵. The penumbra consists of stratified layers, such as the selective cell death zone, heat shock protein 70-inducible zone, hypoxia inducible factor (HIF) zone, and spreading depression zone. In the HIF zone, HIF induces vascular endothelial growth factor (VEGF), iNOS, and erythropoietin. VEGF promotes vascular remodeling, and iNOS increases blood flow by the production of NO. These phenomena cause vascular remodeling in the ischemic penumbra, which

might cause HT through BBB disruption.

Another new definition of an ischemic penumbra is the region of transition from an injury to repair by various mediators⁸ (**Fig. 4**). Interestingly, the factors that are associated with cell death and tissue damage during the acute period might also play roles in tissue recovery in the chronic period. In other words, these mediators have biphasic roles as a harmful and beneficial target in stroke pathophysiology. During the acute phase, most of these targets mediate injury. In contrast, during the recovery phase, the same mediators induce vascular remodeling/angiogenesis and neurogenesis after stroke. New vessels would not be fully matured. Therefore, during this vascular remodeling, vessels are leakier and prone to HT because of vascular unsteadiness⁴⁶. The modulation of remodeling factors after stroke with tPA treatment might be one of the ideal therapeutic strategies to attenuate HT.

The Biphasic Nature of Molecular Signals in the Ischemic Penumbra

Various drugs that attenuate intracerebral HT after tPA treatment have been investigated in experimental animal models (**Table 1**). Interestingly, these therapeutic target molecules, including the NMDA-type glutamate receptor, tumor necrosis factor- α ,

Fig. 4. Ischemic penumbra (modified by reference 8)

The new definition of ischemic penumbra is the transition region from injury to repair (A) and with time course after injury (B).

MMPs, and VEGF, have a biphasic nature⁸. The NMDA-type glutamate receptor is involved not only in the neuronal damage that is mediated by excitotoxicity in the early acute phase but also in neuronal regeneration in the recovery phase⁴⁷. MMPs cause BBB disruption in the acute phase but are essential for angiogenesis/remodeling in the recovery phase. The knockout of genes encoding MMPs and the suppression of selective MMP inhibitors have all proven considerably protective in animal models of stroke²⁶. The degradation of BBB components by MMP causes edema, HT, and neuronal death. In addition, the importance of MMPs is underscored by the fact that they are upregulated by tPA. Thus, these findings suggest that blocking MMPs might attenuate the HTs that currently limit the widespread application of tPA treatment⁴⁸. However, as usual, things are never as simple as we wish them to be. Although MMPs disrupt the neurovascular matrix and cause injury during acute stroke, they can promote neurovascular remodeling in the peri-infarct cortex during the delayed stages of stroke recovery⁴⁹. In addition, MMPs mediate the movement of neuroblasts during the endogenous neurogenic response that is triggered after brain

injury⁵⁰. Whereas the use of MMP inhibitors during the first few hours after stroke reduces infarction, the same inhibitors could worsen the outcomes when they are applied several days later⁵¹.

VEGF also plays a role in the MMP-9-mediated disruption of the BBB in the early acute phase⁵² and in angiogenesis/remodeling in the recovery phase⁵³. Remodeling factors play dual roles in the acute and recovery period. From this point of view, drugs that inhibit cell death in the acute phase and that do not adversely affect the subsequent repair of neuronal cells and blood vessels are preferred.

Vascular Remodeling Factors, Vascular Endothelial Growth Factor, and Angiopoietin-1, as Novel Therapeutic Target Molecules

We identified the remodeling factors, VEGF and angiopoietin-1 (Ang1), as therapeutic target molecules for the prevention of intracerebral HT after tPA treatment^{52, 54}. VEGF induces the proliferation, migration, and enhanced permeability of vascular endothelial cells⁵⁵. The administration of VEGF to

Table 1. Drug candidates to attenuate intracerebral HT after tPA treatment in animal models (modified by reference 58)

Drug candidates	Reference	Animal	Model
MMP inhibitor			
BB-94 (pan-MMP inhibitor)	Sumii <i>et al.</i> Stroke 2002	SHR	eMCAO
Activated protein	Cheng <i>et al.</i> Nat Med 2006	rat	eMCAO
Anti-TNF- α antibody	Lapchak. Brain Res 2007	rabbit	eMCAO
Minocycline	Murata <i>et al.</i> Stroke 2008	SHR	eMCAO
Cilostazol	Ishiguro <i>et al.</i> Plos One 2010	mouse	tMCAO
Anti-VEGF antibody/ receptor inhibitor	Kanazawa <i>et al.</i> JCBFM 2011	rat	eMCAO
Free radical scavenger			
NXY-059	Lapchak <i>et al.</i> Stroke 2002	rabbit	eMCAO
Edaravone	Yamashita <i>et al.</i> JCBFM 2009	SHR	tMCAO
Immunosuppressant			
FK506	Okubo <i>et al.</i> Brain Res 2007	rat	eMCAO
Fingolimod	Campos <i>et al.</i> Stroke 2013	mouse	eMCAO
Statin			
Atorvastatin	Zhang <i>et al.</i> JCBFM 2009	rat	eMCAO
Simvastatin	Lapchak <i>et al.</i> Brain Res 2009	rabbit	eMCAO
Others			
Caffeinol	Aronowski <i>et al.</i> Stroke 2003	rat	tMCAO
Imatinib (PDGFR- α antagonist)	Su <i>et al.</i> Nat Med 2008	mouse	eMCAO
High density lipoprotein	Lapergue <i>et al.</i> Stroke 2013	rat	eMCAO
Insulin	Fan <i>et al.</i> Stroke 2013	rat	eMCAO
Angiopoietin 1	Kawamura <i>et al.</i> PLoS One. 2014	rat	eMCAO
Annexin A2	Jiang <i>et al.</i> Neurosci lett 2015	rat	eMCAO
Bryostatins	Tan <i>et al.</i> Eur J Pharmacol 2015	rat	eMCAO
Gas			
Hyperbaric oxygen therapy	Qin <i>et al.</i> Stroke 2007	rat	tMCAO
Normobaric hyperoxia therapy (100% O ₂)	Liang <i>et al.</i> Stroke 20015	rat	tMCAO
Xenon	David <i>et al.</i> JCBFM 2010	rat	eMCAO

MMP, matrix metalloproteinase; PDGFR- α , platelet-derived growth factor receptor- α ; SHR, spontaneous hypertensive rat; eMCAO, embolic middle cerebral artery occlusion; tMCAO, transient MCAO; TNF- α , tumor necrosis factor- α ; VEGF, vascular endothelial growth factor

animal models in the early phase of acute cerebral ischemia enhances vascular permeability, while the administration in the recovery phase promotes angiogenesis⁵⁶. Employing embolic middle cerebral artery occlusion models, we demonstrated that the VEGF signal cascade is activated at the BBB in the ischemic penumbra, thereby activating MMP-9 and degrading protein components of the basal lamina, which in turn results in HT⁵². These changes were evident when tPA was administered after the therapeutic time window. Moreover, any of the changes, including MMP-9 activation and the degradation of the BBB components, were inhibited by an anti-VEGF neutralizing antibody and receptor antagonist. Thus, we demonstrated that the VEGF signal cascade that is related to tPA treatment is located upstream of

MMP-9 and that VEGF is a promising therapeutic target molecule that is involved in intracerebral HT after tPA treatment (**Fig. 5**)^{52, 57}.

Another endothelial cell-specific growth factor, Ang1⁵⁸, binds to its receptor Tie-2, which is expressed in various types of cells, such as endothelial cells, pericytes, and neuronal cells⁵⁹. Ang1 participates in the survival of endothelial cells, vascular remodeling, and vascular maturation and stability⁶⁰. Ang1 has been reported to reduce the postischemic vascular hyperpermeability that is triggered by VEGF⁶¹. We confirmed that reduced endogenous Ang1 expression was also involved in HT after tPA treatment⁵⁴. We demonstrated that the administration of a recombinant Ang1 protein suppressed HT, as well as cerebral edema, after tPA treatment. Several studies have

Fig. 5. VEGF signaling cascade and anti-VEGF therapy (quoted from reference 57)

After cerebral ischemia, vascular endothelial growth factor (VEGF) is expressed in the microvascular wall, and receptors that are conjugated to VEGF as a ligand are phosphorylated and activated. The subsequent activation of matrix metalloproteinase-9 (MMP-9) and degradation of protein components of the basal lamina cause intracerebral hemorrhage. The VEGF signaling cascade is inhibited by the anti-VEGF antibody that neutralizes VEGF and VEGF receptor inhibitors that inhibit VEGF receptors from being phosphorylated. BBB, blood-brain barrier.

reported that Ang1 suppresses vascular hyperpermeability by increasing glycocalyx in endothelial cells⁶², acting on tight junction proteins⁶³, and working through the signaling of the platelet-derived growth factor-B in pericytes⁶⁴. Future studies are needed to confirm whether Ang1 prevents HT and cerebral edema after tPA treatment by suppressing the permeability that is mediated by platelet-derived growth factor-B signaling in pericytes.

Pleiotropic Mechanisms by Progranulin in Ischemic Stroke

As described above, we have demonstrated that the inhibition of the VEGF signaling pathway and the administration of Ang1 attenuates HT after tPA treatment of ischemic stroke. Although this treatment can enable vascular protection, it cannot reduce the cerebral infarct volume^{52, 54} because it does not have neuroprotective or anti-inflammatory effects. We suggest that brain protection, which includes vascular protection, neuroprotection, and anti-inflammation, is an

ideal therapeutic strategy for ischemic stroke. We identified a target molecule, progranulin (PGRN) (Fig. 6)⁶⁵. In the central nervous system, PGRN is a growth factor that is thought to play crucial roles in maintaining physiological functions⁶⁶ because mutations of the *PGRN* gene cause the familial dementia, TAR DNA binding protein-43 (TDP-43)-positive frontotemporal lobar degeneration⁶⁷⁻⁶⁹. We have reported nuclear the TDP-43 might be involved in neuronal cell death prior to cell death after cerebral ischemia^{65, 70}. We have demonstrated dynamic changes in PGRN expression, including increased levels of PGRN expression in microglia within the ischemic core and in surviving neurons, as well as the induction of PGRN expression in endothelial cells within the ischemic penumbra, in ischemic rats. We have observed that PGRN protects against acute focal cerebral ischemia through brain protection, including neuroprotection that occurs in part by the inhibition of the cytoplasmic redistribution of nuclear TDP-43, suppression of neuroinflammation through anti-inflammatory interleukin-10 in microglia, and attenu-

Fig. 6. The pleiotropic effects of the brain protective target progranulin (PGRN)

The growth factor PGRN could protect against acute focal cerebral ischemia through a variety of mechanisms, which we call brain protection. The nuclear protein TAR DNA binding protein-43 (TDP-43) is localized in the nucleus. However, cytoplasmic redistribution of TDP-43 occurs after ischemia. Intravenously administered recombinant PGRN significantly reduced the cerebral infarct and edema volumes, suppressed hemorrhagic transformation, and improved motor outcome in thromboembolic rats with delayed tissue plasminogen activator (tPA) treatment because of neuroprotection that occurred in part through the inhibition of the cytoplasmic redistribution of TDP-43, suppression of neuroinflammation through anti-inflammatory interleukin-10 (IL-10) in microglia, and attenuation of BBB disruption through the vascular endothelial growth factor (VEGF). PGRN might be a novel therapeutic target that provides brain protection through processes, such as vascular protection, anti-neuroinflammation, and neuroprotection.

ation of BBB disruption through the inhibition of VEGF. Finally, intravenously administered recombinant PGRN significantly reduces the volumes of cerebral infarcts and edema, suppresses HT, and improves motor outcomes in thromboembolic rats with delayed tPA treatment⁶⁵. Several other researchers have also shown the pleiotropic protective effects of PGRN⁷¹⁻⁷³. PGRN might be a novel therapeutic target that provides brain protection.

Next-Generation Therapeutic Strategies

A very recent meta-analysis of randomized trials of endovascular treatment with alteplase showed that the therapeutic time window was 6 h after the onset of stroke⁷⁴. According to the guidelines, patients who are eligible for intravenous tPA should receive intravenous tPA, even if endovascular treatments are being considered (Class I; Level of Evidence A). Moreover, patients should receive endovascular treatment with a

stent retriever if they meet the criterion of being within 6 h after onset of stroke (Class I; Level of Evidence A). These suggestions consider the therapeutic limitations of only alteplase and alteplase with endovascular treatments within 6 h after the onset of stroke. Indeed, the need for the development of protective agents for ischemic stroke was discussed at the 2015 International Stroke Conference in Nashville, TN. The next-generation therapeutic strategies are the following: 1) the selection of eligible patients, 2) development of new thrombolytic agents, and 3) combination treatments with protective agents and only alteplase and alteplase with endovascular treatments.

To extend the therapeutic time window of tPA for the selection of eligible patients, a multicenter, randomized, double-blinded, and placebo-controlled Phase III study to investigate EXtending the time for Thrombolysis in Emergency Neurological Deficits (EXTEND) study⁷⁵ and the European Cooperative Acute Stroke Study-4: Extending the time for throm-

bolysis in emergency neurological deficits (ECASS-4:ExTEND) are ongoing. These studies deal with ischemic stroke patients with diffusion-weighted image and perfusion-weighted image mismatch in patients 4.5 to 9 h after stroke onset. The final results are not yet available.

Some Recent New Thrombolytic Agents

Desmoteplase (*Desmodus rotundus* salivary plasminogen activator) is a desirable and attractive alternative to alteplase, and it has several theoretical advantages. It has demonstrated minimal neurotoxicity, high selectivity, specificity for fibrin, and a long half-life. However, a randomized, placebo-controlled, phase-III clinical trial (DIAS-3) that enrolled AIS patients presenting within 3–9 h of onset showed that the frequency of symptomatic intracranial hemorrhage was the same between the desmoteplase group and placebo groups⁷⁶. The treatment with desmoteplase did not cause safety concerns and did not improve functional outcome when given to patients with ischemic stroke beyond 3 h of onset.

Tenecteplase is a genetically engineered variant of tPA that has a longer half-life and is more fibrin-specific than tPA. These properties give tenecteplase more complete clot lysis with less bleeding complications. In the tenecteplase versus alteplase treatment for patients with AIS who were within 6 h after onset of the ischemic stroke, the tenecteplase group had greater reperfusion rates and better clinical improvement at 24 h compared with the tPA group⁷⁷. Evaluations of tenecteplase in larger trials of patients with acute stroke are warranted because randomized controlled phase-III trials are lacking. The Norwegian Tenecteplase Stroke Trial (NOR-TEST) is ongoing to compare the efficacy and safety of tenecteplase vs. alteplase in larger groups of patients⁷⁸.

Another next-generation thrombolytic agent, Stachybotrys microspora triphenyl phenol-7 (SMTP-7), might be another ideal candidate agent. SMTP-7 was discovered from the fungus *Stachybotrys microspora*. SMTP-7 promotes the urokinase-catalyzed conversion of plasminogen to plasmin, fibrin binding to plasminogen, and the enhancement of thrombolysis in focal ischemic models of rodents and primates. Notably, SMTP-7 suppressed neuroinflammation after reperfusion through the suppression of proinflammatory cytokines^{79, 80}. The results indicated that SMTP-7 decreases infarct volume, HT, mortality, and neurological deficits, and it may be a safe thrombolytic agent to use following cerebral ischemia under warfarin-treated conditions⁸¹.

Combination Treatments with Protective Agents

Clinical trials should be performed on thrombolytic therapies and/or endovascular treatments plus concomitant drugs. The Albumin in Acute Ischemic Stroke (ALIAS) parts 1 and 2 trials evaluated whether 25% human serum albumin improved clinical outcomes after acute ischemic stroke⁸². During the trial, there was a rising use of both intravenous thrombolysis and endovascular stroke treatment. Albumin increased the risk of symptomatic intracerebral hemorrhage in combination with thrombolysis (intravenous and endovascular), although the absolute risk increase was too small to account for the difference between the treatment groups. The ALIAS trials are the latest in a string of clinical trials of putative neuroprotection that have failed to demonstrate clinical efficacy, despite strong preclinical evidence. The vast majority of preclinical models of so-called neuroprotective agents have shown efficacy in models of ischemia-reperfusion. Yet, in humans, on average, early reperfusion was achieved much less than 50% of the time. However, reperfusion was not commonly evaluated. We look forward to new studies that will reexamine the neuroprotection hypothesis in an era of proven early reperfusion⁸³. The glycoprotein IIb/IIIa receptor antagonists (GPIs) in terms of platelet inhibition eptifibatide have demonstrated that AIS patients with tPA plus eptifibatide showed lower incident rates of symptomatic HT than those with tPA alone. The comparison outcomes in patients with tPA and eptifibatide were better than tPA only subjects in ALIAS Part 2 and Interventional Management of Stroke III⁸⁴. A phase III trial to establish the efficacy of tPA plus eptifibatide for improving AIS outcomes is warranted.

In addition, clinical trials with tPA have been conducted with substances, such as atorvastatin, and edaravone, although a very recent study did not show that simvastatin plus tPA combination treatment suppress HT and improve outcome⁸⁵. The trials with interventions need a large number of patients and adequate timing for the intervention not to prevent recovery from the point of view of the new ischemic penumbra. Therapeutic agents with pleiotropic protective mechanism are ideal. Clinical trials which evaluate the effects of anti-VEGF drugs or PGRN-based treatment with tPA will be might worthwhile.

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Conflict of Interest

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