Therapeutic strategies to attenuate hemorrhagic transformation after tissue plasminogen activator treatment for acute ischemic stroke

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Abstract
Here, we describe a therapeutic strategy for attenuating hemorrhagic transformation (HT) after tissue plasminogen activator (tPA) treatment for acute ischemic stroke. Recent studies have shown that tPA treatment is beneficial within 4.5 h of onset for patients with acute ischemic stroke. However, the risk of serious or fatal symptomatic hemorrhage increases with delayed initiation of treatment. HT is considered to be caused by ischemic/reperfusion injury, as well as the toxicity of tPA itself. Therapeutic strategies to attenuate HT after tPA treatment might involve (i) identification of risk factors for HT after tPA treatment and (ii) the development of thrombolytic drugs, which are less likely to cause bleeding, or drugs that can be concomitantly administered for vascular protection. Several studies have shown that matrix metalloproteinases and free radicals are potential therapeutic targets. In addition, we recently showed that inhibition of the vascular endothelial growth factor (VEGF) signaling pathway might be a promising therapeutic strategy for attenuating HT after tPA treatment. Further studies are required to link the results obtained in experimental animal models to human clinical trials.

Introduction
Tissue plasminogen activator (tPA), a thrombolytic drug used for treating acute ischemic stroke, is a grade A recommendation in Japanese and USA guidelines. However, tPA does not always significantly contribute to the treatment of ischemic stroke primarily because of its temporal limitations, wherein tPA treatment should be initiated within 4.5 h of stroke onset and possible intracerebral hemorrhagic transformation (HT) after tPA treatment. Experimental animal model studies have shown that reperfusion by thrombolysis at an inappropriate time increases the intracerebral HT incidence.1 Furthermore, tPA itself is neurotoxic, which aggravates damage caused by glutamic acid release after ischemia. In addition, it promotes infiltration of leukocytes and production of free radicals after ischemic lesions.2 Herein, we review therapeutic strategies aimed at attenuating intracerebral HT after tPA treatment.

Strategies to improve outcomes of tPA treatment
Reperfusion by thrombolysis is essential for the treatment of acute ischemic stroke; however, this treatment alone is not sufficient to restore blood flow. Although thrombolysis efficiency varies depending on the modalities of tPA treatment (intravenous < intra-arterial < combined intravenous/intra-arterial < combined with mechanical thrombolysis),3 there are differences between the efficiency and therapeutic effects of thrombolysis.4 Thus, it seems difficult to prevent the cascade of ischemic neuronal damage with reperfusion alone. The possible events that can occur after reperfusion are summarized in Table 1.

Potential strategies to improve tPA treatment outcomes could involve: (i) reduction in the delay in initiating tPA treatment; (ii) improvement in the efficiency of thrombolysis (recanalization rate); (iii) extension of the therapeutic time window; (iv) reduction in the inherent neurotoxicity of tPA; and (v) attenuation of intracerebral HT (Table 2).

Characteristics of intracerebral HT after tPA treatment
According to a postmarketing survey, the frequency of symptomatic HT after tPA treatment is 8.5% (out of 6,483 patients) in the Safe Implementation of Thrombolysis in Stroke Monitoring Study (SITS-MOST) in Europe,5,6 5.8% (out of 103 patients) in Alteplase Clinical Trial (J-ACT) in Japan,7 and 0% (out of 58 patients) in J-ACT II using magnetic resonance angiography.8 If the treatment is initiated
Table 1 Possible events after reperfusion

| Events                                                                 |
|----------------------------------------------------------------------|
| 1. Cerebral edema and intracerebral hemorrhage associated with disruption of the blood-brain barrier |
| 2. Induction of cell death processes (e.g. apoptosis, necrosis, and autophagy-related death) |
| 3. Reprogramming of transcription                                      |
| 4. Activation of innate and adaptive immunity                          |
| 5. No reflow phenomenon                                                 |

*A phenomenon in which, if a certain period of time elapses after stroke onset, restoration of blood flow is not expected even after reperfusion of the ischemic lesion or hypoperfusion after temporary hyperperfusion.

Table 2 Strategies to improve thrombolytic treatment outcomes

| Therapeutic strategy | Specific methods |
|----------------------|------------------|
| (1) Reduction in time before treatment initiation | Awareness campaign for stroke | Reexamination of patient transport methods and treatment after arrival at hospital |
| (2) Improvement in efficiency of thrombolysis | Examination of administration methods of tPA | Concomitant drugs (e.g. selective thrombin inhibitors, low-molecular-weight heparin, aspirin, and glycoprotein IIb/IIIa inhibitors) |
| (2) Improvement in efficiency of thrombolysis | Concomitant use of transcranial ultrasound (Combined Lysis of Thrombus in Brain Ischemia with Transcranial Ultrasound and Systemic tPA [CLOTBUST] trial) |
| (3) Extension of the therapeutic time window | Selection of patients eligible for treatment using imaging techniques | Development of concomitant drugs that protect the penumbra |
| (4) Reduction in inherent tPA neurotoxicity | Reduction in tPA doses | Development of novel thrombolytic drugs (e.g. alteplase and desmoteplase) |
| (5) Attenuation of intracerebral HT | Identification of high-risk patients | Development of novel thrombolytic drugs |
| (5) Attenuation of intracerebral HT | Development of concomitant vasoprotective drugs |

* tPA, tissue plasminogen activator, HT, hemorrhagic transformation.

Mechanism of intracerebral HT after tPA treatment

Major causes for disruption of the blood-brain barrier (BBB), which is involved in intracerebral HT after tPA treatment, include: (i) cerebral ischemia/reperfusion injury; and (ii) the inherent toxicity of tPA. Cerebral capillaries have a vascular structure composed of vascular endothelial cells and their tight junction, pericytes, basal lamina, and astrocytes surrounding them. This characteristic structure plays an important role maintaining BBB function. Cerebral ischemia/reperfusion injury results in: (i) the disappearance of the vascular permeability barrier; (ii) degradation of protein components of the basal lamina by matrix metalloproteinase (MMP)-2 or MMP-9 followed by detachment of the basal lamina and astrocytes; and (iii) infiltration of inflammatory cells into the vascular wall in the BBB.

Furthermore, the inherent action of tPA can induce intracerebral HT through mechanisms involving: (i) prolonged bleeding times; (ii) plasmin-mediated degradation of protein components of the basal lamina; (iii) production of free radicals associated with reperfusion; and (iv) MMP activation. Through interaction with specific domains of proteins, tPA causes excitatory neurotoxicity, activation of platelet-derived growth factor-CC (PDGF-CC), and activation of microglia (Fig. 2). There is a report that caspase-8-mediated apoptosis is involved in tPA-mediated neurotoxicity.

Regarding the activation of MMP, it has been shown that tPA cleaves low-density lipoprotein (LDL) receptor-related protein (LRP) in the plasma membrane of astrocytes that exist around blood vessels, and the cleaved extracellular fragments induce MMP-9 through the nuclear factor (NF)-κB pathway activation. Table 3 shows the roles of typical MMP in cerebral ischemia.

Strategies to attenuate intracerebral HT after tPA treatment

Possible strategies to attenuate intracerebral HT after tPA treatment include: (i) identification of patients at high risk for the development of intracerebral HT and selection of candidates for tPA treatment; (ii) reduction of tPA doses; and (iii) development of thrombolytic and vasoprotective drugs that are unlikely to cause hemorrhage. For strategy (i), advanced age, severe cases, hypertension, and atrial fibrillation, as well as hyperglycemia, which promotes superoxide production, are recognized as important predictors for...
intracerebral HT.\textsuperscript{6,19} Plasma MMP-9\textsuperscript{20,21} and fibronectin,\textsuperscript{21} the substrate of MMP-9, are biomarkers for HT in humans. Imaging studies are also useful to predict intracerebral HT. The Alberta Stroke Program Early Computed Tomography Score (ASPECTS) is a quick and standardized CT scoring system.\textsuperscript{22} For this score, the territory supplied by the middle cerebral artery is allotted 10 points, and 1 point is subtracted for each area of early ischemic change for each of

### Table 3 Matrix metalloproteinases and their putative roles in acute ischemic stroke

| MMP              | Putative roles                                                                 |
|------------------|-------------------------------------------------------------------------------|
| MMP-2 (gelatinase A) | Attacks major components of the basal lamina around the cerebral blood vessels including type IV collagen, laminin, and fibronectin. Could contribute to infarct and hemorrhagic volume. |
| MMP-3 (stromelysin-1) | Degrades the extracellular matrix proteins fibronectin, denatured collagen, laminin, and proteoglycans. Plays a key role in the initial opening of the BBB after stroke and development of hemorrhagic transformation, particularly with tPA treatment. |
| MMP-9 (gelatinase B) | Attacks major components of the basal lamina including type IV collagen, laminin, and fibronectin. Plays a key role in the delayed opening of the BBB after stroke especially in states of systematic inflammation. Implicated in the development of hemorrhagic transformation, particularly with tPA treatment. |

BBB, blood-brain barrier; MMP, matrix metalloproteinase; tPA, tissue plasminogen activator. Modification of reference\textsuperscript{17}.  

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**Figure 1** The association between parenchymal intracerebral hemorrhage and the time to initiation of tissue plasminogen activator treatment (adapted from Lees KR \textit{et al} \textsuperscript{9}). The horizontal axis is the time to initiate treatment (min), and vertical axis is odds ratio and 95% confidence interval for type-II parenchymal intracerebral hemorrhage (Stroke 2001; \textbf{32}:1330–5133).

**Figure 2** Mechanisms of intracerebral hemorrhagic transformation after tissue plasminogen activator (tPA) treatment and therapeutic targets. APC, activated protein C; BBB, blood-brain barrier; LRP, Low-density lipoprotein receptor-related protein; MMP, matrix metalloproteinase; NMDA, N-methyl-D-aspartate; PAR1, protease activated receptor 1; tPA, tissue plasminogen activator; VEGF, vascular endothelial growth factor.
the defined regions (cut-off value for HT ≤ 7). Diffusion-weighted imaging (DWI)-ASPECTS is a scoring method for assessing the presence of fresh ischemic lesions by using DWI compared with CT-ASPECTS (cut-off value for HT ≤ 5 out of 10 points). ASPECTS + W is a modified ASPECTS for DWI, which includes deep white matter lesions on DWI in addition to the original ASPECTS regions (cut-off value for HT ≤ 7 out of 11 points). In addition, the selection of patients by using diffusion perfusion mismatch on DWI ("malignant profile", characterized by a large DWI lesion volume and/or a large perfusion-weighted image lesion volume with long delays on the Tmax map, as associated with a high risk for fatal symptomatic hemorrhage) or new-extra ischemic microbleeds developed rapidly after tPA treatment on T2*WI or susceptibility-weighted image (SWI) might decrease intracerebral HT after tPA treatment.

The usefulness of the SEDAN score (sugar on admission, early infarct signs and [hyper] dense cerebral artery sign on admission CT head scan, age, and the National Institutes of Health Stroke Scale [NIHSS] score), which is used to estimate risk of symptomatic intracerebral HT by combining these predictors, has been reported (Table 4a). Other predictive scores such as HAT, SITS-ICH risk score, and postthrombolysis risk score have also been proposed (Table 4b).

It has also recently been reported that high signal intensity lesions on fluid-attenuated inversion recovery imaging carried out within 4.5 h after stroke onset and very low cerebral blood volume on the cerebral blood flow map created by perfusion imaging are useful for predicting intracerebral HT.

For strategy (ii), the single-arm case-controlled observational studies showed that the clinical efficacy and safety of low-dose intravenous (IV)-tPA (0.6 mg/kg body weight; maximum 60 mg) in Japanese patients with acute ischemic stroke patients is comparable with those of thrombolysis with standard-dose IV tPA (0.9 mg/kg body weight; maximum 90 mg) in Western population.

Regarding strategy (iii), various drugs that attenuate intracerebral HT after tPA treatment have been investigated in experimental animal models, and MMP were frequently used as therapeutic target molecules in these studies (Table 5). Specifically, the aim of these studies was to inhibit MMP that are possibly released from neutrophils and microglia that cause destruction of tight junctions that connect the basal lamina and endothelial cells. The importance of MMP-9 is shown by the findings that

### Table 4 Prediction of symptomatic intracerebral hemorrhage risk: (a) The SEDAN score. (b) Scores predicting the risk of hemorrhagic transformation after thrombolysis

| Item | Value | Score |
|------|-------|-------|
| a) The SEDAN score | | |
| Blood sugar | ≤ 144 mg/dL | 0 |
| | 145-216 mg/dL | 1 |
| | > 216 mg/dL | 2 |
| Early ischemic signs on CT | Absent | 0 |
| | Present | 1 |
| Hyperdense artery sign | Absent | 0 |
| | Present | 1 |
| Age | ≤ 75 years | 0 |
| | > 75 years | 1 |
| NIH Stroke Scale score | 0-9 points | 0 |
| | ≥ 10 points | 1 |

| Component | Risk assessment |
|-----------|-----------------|
| SEDAN age, NIHSS score, HDMCA signs, early infarct signs on CT, glucose at presentation | Increased risk of ICH for scores 0–6 |
| HAT DM or glucose at presentation ≥ 200 mg/dL, NIHSS score, hypodensity on CT | Increased risk of ICH for scores 0–5 |
| SITS-ICH Risk Score age, body weight, history of hypertension, use of aspirin/clopidogrel, NIHSS score, SBP, glucose at presentation, OTT | Increased risk of ICH for scores 0–12 |
| Postthrombolysis Risk Score age > 60 years, NIHSS > 10, glucose > 8.325 mmol/L, platelets < 150 000/mm³ | Increased risk of ICH for scores 0–4 |

*Sugar on admission, early infarct signs and (hyper) dense cerebral artery sign on admission CT head scan, age, and the National Institutes of Health Stroke Scale (SEDAN) scores range from 0–6 points. The absolute risk of symptomatic intracerebral hemorrhage is 1.0–1.4% for 0 point, 2.9–3.5% for 1 point, 5.1–8.5% for 2 points, 9.2–12.2% for 3 points, 16.9–21.7% for 4 points, and 27.8–33.3% for 5 points (refer to reference29). DM, diabetes mellitus; HAT hemorrhage after thrombolysis; HDMCA, hyperdense middle cerebral artery; ICH, intracranial hemorrhage; NIHSS, National Institutes of Health Stroke Scale; OTT, onset of symptoms to treatment; SBP, systolic blood pressure; SITS-ICH, Safe Implementation of Treatments in Stroke-symptomatic Intracerebral Hemorrhage Modification of reference.55
**Table 5** Drug candidates

| Drug                          | Reference                                      | Animal | Model    |
|-------------------------------|------------------------------------------------|--------|----------|
| **MMP inhibitors**            |                                                |        |          |
| BB-94 (pan-MMP inhibitor)     | Sumii *et al.* Stroke, 2002                    | SHR    | eMCAO    |
| Activated protein             | Cheng *et al.* Nat Med, 2006                   | Rat    | eMCAO    |
| Anti-TNFα antibody            | Lapchak *et al.* Brain Res, 2007               | rabbit | eMCAO    |
| Minocycline                   | Murata *et al.* Stroke, 2008                   | SHR    | eMCAO    |
| AHA (pan-MMP inhibitor)       | Copin *et al.* Exp Neurol, 2008                | Rat    | eMCAO    |
| Proteasome inhibitor          | Zhang *et al.* Stroke, 2010                    | Rat    | eMCAO    |
| Cilostazol                    | Ishiguro *et al.* Plos One, 2010               | Mouse  | tMCAO    |
| Anti-VEGF antibody/receptor inhibitor | Kanazawa *et al.* JCBFM, 2011              | Rat    | eMCAO    |
| Fasudil (rho kinase inhibitor)| Ishiguro *et al.* Neuroscience, 2012          |        |          |
| **Free radical scavengers**   |                                                |        |          |
| NXY-059                       | Lapchak *et al.* Stroke, 2002                  | Rabbit | eMCAO    |
| Edaravone                     | Yamashita *et al.* JCBFM, 2009                 | SHR    | tMCAO    |
| Melatonin                     | Chen *et al.* J Pineal Res, 2006               | Mouse  | eMCAO    |
| **Immunosuppressants**        |                                                |        |          |
| FK506                         | Okubo *et al.* Brain Res, 2007                 | Rat    | eMCAO    |
| Fingolimod                    | Campos *et al.* Stroke, 2013                   | Mouse  | eMCAO    |
| **Statins**                   |                                                |        |          |
| Atorvastatin                  | Zhang *et al.* JCBFM, 2009                     | Rat    | eMCAO    |
| Simvastatin                   | Lapchak *et al.* Brain Res, 2009               | Rabbit | eMCAO    |
| **Others**                    |                                                |        |          |
| Caffeineol                    | Aronowski *et al.* Stroke, 2003                | Rat    | tCCA/MCAO|
| Imatinib (PDGFR-α antagonist) | Su *et al.* Nat Med, 2008                      | Mouse  | eMCAO    |
| High density lipoprotein      | Lapergue *et al.* Stroke, 2013                 | Rat    | eMCAO/tMCAO|
| Insulin                       | Fan *et al.* Stroke, 2013                      | Rat    | eMCAO    |
| **Gases**                     |                                                |        |          |
| Hyperbaric oxygen therapy     | Qin *et al.* Stroke, 2007                      | Rat    | tMCAO    |
| Normobaric oxygen therapy(95% O₂) | Liu *et al.* Stroke, 2009              | Rat    | tMCAO    |
| Xenon                         | David *et al.* JCBFM, 2010                     | Rat    | eMCAO    |

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

![Figure 3](https://image-url.com)  
**Figure 3** Vascular endothelial growth factor (VEGF) signal cascade and anti-VEGF therapy. After cerebral ischemia, VEGF are expressed in the microvascular wall, and receptors conjugated to VEGF as a ligand are phosphorylated and activated. Subsequent activation of matrix metalloproteinase (MMP-9) and degradation of protein components in the basal lamina cause intracerebral hemorrhage. VEGF signaling is inhibited by the anti-VEGF antibody that neutralizes VEGF as well as by VEGF receptor inhibitors that inhibit VEGF receptors from being phosphorylated.

pathological examination of the brains from patients who died of intracerebral HT after tPA treatment revealed accumulation of MMP-9-positive neutrophils in microvessels\(^22\) and that disruption of the BBB is unlikely to be observed after cerebral ischemia in MMP-9 knockout (KO) mice.\(^41\) Regarding the mechanism underlying MMP-9 activation,
the roles of tPA and free radicals are emphasized. A previous study has shown that in tPA KO mice, cerebral infarct size was smaller and cerebral edema was milder as compared with wild-type mice, and the mechanism involved lack of MMP-9 activity after cerebral ischemia. The aforementioned LRP and protease-activated receptor 1 (PAR1) are involved in pathways through which tPA activates MMP-9 (Fig. 2). The LRP inhibitor targets LRP, and activated protein C (APC) targets PAR1. Furthermore, MMP-3 is involved in the occurrence of intracerebral HT. It has also been reported that intracerebral HT is unlikely to occur after tPA treatment in MMP-3 KO mice, and that LRP is associated with MMP-3 activation after tPA treatment (Fig. 2).

In addition, focal cerebral ischemia models of mice deficient for superoxide dismutase (SOD) 2, an oxidation reduction enzyme, showed increased MMP-9 activation and intracerebral HT, suggesting that production of free radicals activates MMP-9.

**Vascular endothelial growth factor as a novel therapeutic target molecule**

We identified vascular endothelial growth factor (VEGF) as a therapeutic target molecule for intracerebral HT after tPA treatment. VEGF induces proliferation, migration, and enhanced permeability of vascular endothelial cells. Administration of VEGF to animal models in the early phase of acute cerebral ischemia enhances vascular permeability, whereas administration in the recovery phase promotes angiogenesis. By using embolic middle cerebral artery occlusion models, we showed that VEGF signaling is activated at the BBB in the marginal zone of the ischemic lesion after ischemia, thereby activating MMP-9 and degrading protein components of the baslamina, and thereby resulting in intracerebral hemorrhage. These changes were noticeable when tPA was administrated after the therapeutic time window. Furthermore, all changes involving MMP-9 were inhibited using the anti-VEGF neutralizing antibody or receptor antagonist. Thus, it appears that the VEGF signaling cascade is located upstream of MMP-9, and VEGF is a promising therapeutic target molecule for intracerebral HT after tPA treatment (Fig. 3).

**Dual nature of therapeutic target molecules**

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