Annexin A2
A Tissue Plasminogen Activator Amplifier for Thrombolytic Stroke Therapy
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Abstract—Hemorrhagic transformation, incomplete reperfusion, neurotoxicity, and the short treatment time window comprise major challenges for thrombolytic therapy. Improving tissue plasminogen activator therapy has become one of the highest priorities in the stroke field. Recent efforts have been aimed at identifying new strategies that might enhance the thrombolytic efficacy of tissue plasminogen activator at the same time as reducing its associated complications related to hemorrhage and neurotoxicity. We believe that the combination of low-dose tissue plasminogen activator with recombinant annexin A2 (a tissue plasminogen activator and plasminogen coreceptor) might constitute a promising approach. Our pilot study using a focal embolic stroke model in rats supports this hypothesis. (Stroke. 2010; 41[suppl 1]:S54-S58.)

Key Words: annexin A2 combination therapy cerebral ischemia thrombolysis tPA

Recent clinical investigations have demonstrated the potential for improving tissue plasminogen activator (tPA) therapy. For instance, perfusion-weighted imaging–diffusion-weighted imaging mismatch in MRI studies suggest that some patients may benefit from treatment beyond the conventional 3-hour time window. European Cooperative Acute Stroke Study (ECASS) III, a randomized Phase III trial designed to test treatment with tPA at 3 to 4.5 hours, showed that intravenous tPA given during this time window still improved clinical outcomes in patients with somewhat milder stroke scores. However, there was still a large difference in ORs between early reperfusion within 90 minutes (approximately 2.8 OR) versus delayed reperfusion (approximately 1.4 OR). Thus, the benefits of thrombolysis are still heavily dependent on time to treatment, and use of tPA may still be associated with intracranial hemorrhage and reperfusion injury. It is, therefore, imperative that we seek combination therapies that will truly broaden the therapeutic window, reduce the risk of tPA-associated hemorrhagic transformation, and enhance thrombolytic efficacy. For this purpose, we believe that the combination of low-dose tPA with recombinant annexin A2 protein (rA2) may be promising.

Pleiotropic Effects of tPA Reperfusion Treatment
Ischemic stroke is a cerebrovascular event. In acute ischemic stroke, tPA thrombolytic therapy is intended to reopen occluded vessels by lysis of the thrombus, thereby improving clinical outcome through regional reperfusion and salvage of threatened tissues. tPA acts primarily inside the blood vessel; if blood flow is successfully restored by early thrombolytic reperfusion, then compromised brain tissue can be rescued. However, what happens when tPA thrombolysis is not fully successful as occurs in approximately 50% of patients and reperfusion occurs within the context of weakened vessels and perturbed neurovascular homeostasis? Although unequivocal human data are lacking, findings from animal models suggest that tPA reperfusion may have deleterious consequences due to the nonthrombolytic actions of tPA. Although more investigations are required to dissect the molecular signaling mechanisms initiated by exogenous tPA in the occluded vessel and ischemic brain, emerging data suggest that, besides thrombolysis per se, exogenous tPA may have additional pleiotropic actions within the brain. These may include direct vasoactivity, enhanced excitotoxicity, and activation of extracellular proteases such as matrix metalloproteinases that may promote neurovascular injury. These actions of tPA may increase ischemic neurotoxicity, damage the blood–brain barrier, and exacerbate edema and cerebral hemorrhage, thereby compromising its usefulness as a thrombolytic agent.

Recanalization, moreover, is an important predictor of stroke outcome regardless of thrombolytic modality used.
Importantly, exogenous tPA may worsen ischemia-induced blood–brain barrier disruption, elevate the risk of symptomatic intracranial hemorrhage, and thus reduce the therapeutic time window. Although 1 strategy to overcome these dose-dependent side effects of tPA might be simply to lower the tPA dose, this step would be likely to lower perfusion efficacy. Clearly, optimization of tPA-induced thrombolysis requires that one balance the potential benefits of reperfusion against the detrimental effects of exogenous tPA. Improving tPA thrombolytic regimens may both lengthen the time-to-treatment window and make reperfusion therapy safer and more efficacious.12,13 Recent efforts have been aimed at identifying new combination strategies that might increase the thrombolytic efficacy of tPA at the same time as reducing its associated neurotoxicity and hemorrhagic transformation.9,14–18

### tPA Receptor Annexin A2 and Fibrinolytic Assembly

In fibrinolysis, tPA serves a key role in regulating the breakdown of fibrin-containing thrombi by enzymatically converting clot-bound plasminogen to active plasmin. Plasmin, in turn, degrades crosslinked fibrin and, possibly, intact fibrinogen, a process called fibrinogenolysis.19 However, recent vascular biology studies have revealed that tPA interacts with cellular receptors that allow it to carry out additional biological functions and to activate specific signal transduction pathways.20 A central tenet of cell surface fibrinolysis is the concept of fibrinolytic assembly, in which the tPA-dependent conversion of plasminogen to active plasmin is precisely orchestrated through the formation of a multicomponent complex consisting of tPA, the annexin A2 heterotetramer, and plasminogen (Figure 1).21

Annexin A2 is a cell-surface protein, which, in complex with its binding partner p11, forms a heterotetrameric \((A_2p11)_2\) receptor for both plasminogen, the inactive precursor of plasmin, and its activator, tPA. By assembling tPA, annexin A2, and plasminogen, this complex increases the catalytic efficiency of tPA, enabling it to convert plasminogen to plasmin at least 60 times more efficiently than the same amount of tPA alone.21,22 Expressed by endothelial cells, annexin A2 exists in both membrane-bound and soluble forms,23 and it can be transported to the cell surface in response to cellular stress.24

Fibrinolytic assembly plays a critical role in maintaining blood and vascular homeostasis.25 Interestingly, complete deficiency of annexin A2 in mice leads to a lack of tPA cofactor activity, accumulation of intravascular fibrin, and failure to clear arterial thrombi.26 In addition, earlier studies demonstrated that soluble rA2 reduces thrombus formation in rat carotid27 and middle cerebral arteries in vivo.28 These studies indicate that the use of rA2 for enhancing tPA thrombolytic efficiency may be feasible.

### Combination Stroke Therapy of rA2 Protein Plus Low-Dose tPA

As noted, it has been demonstrated that tPA converts plasminogen to clot-dissolving plasmin and that this action is enhanced by complex formation among tPA, annexin A2, and plasminogen. However, clinical experience has shown that treatment with high doses of exogenous tPA alone may be partially responsible for the limitations of tPA reperfusion stroke therapy. Increased risk of hemorrhage, lower reperfusion efficiency, and the abbreviated therapeutic time window associated with higher-dose tPA constitute major challenges in this field.9,29 Our overall hypotheses have been that rA2 will reduce the dose of tPA required for reperfusion at the same time as enhancing thrombolytic efficacy and attenuating intracerebral hemorrhagic transformation. We postulate further that the combination of tPA and rA2 will lengthen therapeutic time windows and improve long-term outcomes. We have tested these hypotheses in a pilot study. For this purpose, we synthesized and purified rA2 as previously described.27 Consistent with previous reports,21,30 in vitro plasmin activity assays showed that rA2 significantly amplified tPA-mediated plasmin generation.31 We also found that equivalent levels of plasmin activity can be reached by using high-dose tPA alone or lower-dose tPA in combination with...
The simultaneous use of tPA with rA2 at approximately a 1:2 wt/vol ratio (or 1:4 molar ratio) increased the plasmin-generating capability of tPA in vitro by almost 4-fold (Figure 2). We then tested our hypothesis in a focal embolic stroke model in rats. This model was induced by injection of 1 blood clot (40 mm in length) into the middle cerebral artery as we previously described.31 Because of species-related differences in fibrin specificity, the equivalent effective dose of human recombinant tPA in the rat was approximately 10 times higher than the dose used in humans, or approximately 10 mg/kg.32 In the first set of experiments, animals were treated intravenously 2 hours after initiation of ischemia. Neither intermediate (5 mg/kg) nor low (2.5 mg/kg) doses of tPA alone nor rA2 (5 mg/kg) alone was effective in improving reperfusion or reducing infarction. However, the combination of low-dose tPA (2.5 mg/kg) plus rA2 (5 mg/kg) was as effective as the standard high-dose tPA alone in restoring perfusion and reducing infarct size.31 These data suggested that rA2 can make low-dose tPA more effective in an embolic stroke animal model.

In the second set of experiments, rats were treated intravenously in a delayed fashion, 4 hours after the onset of stroke, with saline, high-dose tPA (10 mg/kg), or low-dose tPA (2.5 mg/kg) plus rA2 (5 mg/kg). At 24 hours after the initiation of stroke, the combination of low-dose tPA (2.5 mg/kg) plus rA2 (5 mg/kg) significantly reduced infarct volume when compared with either saline or high-dose tPA alone.31 As expected, high-dose tPA administered at the delayed 4-hour time point induced significant hemorrhagic transformation, whereas hemorrhage was significantly ameliorated by the combination regimen (Figure 3).

To test whether the combination of tPA and rA2 can improve neurological outcomes in longer survival time after stroke, rats were treated in a third experiment in a delayed fashion, 4 hours after stroke, with either saline or a combination of low-dose tPA (2.5 mg/kg) plus rA2 (5 mg/kg). At 3 days after stroke, the combination regimen was found to significantly decrease brain infarct volume (Figure 4A) and neurological deficits (Figure 4B). Overall mortality, furthermore, was significantly reduced in the combination therapy group (21% [3 of 14]) in comparison with saline-treated animals (42% [6 of 14]). Taken together, these results demonstrate that addition of the “tPA amplifier” rA2 may reduce the effective thrombolytic dose of tPA, minimize hemorrhage and brain infarction, and prolong the reperfusion time window in stroke. These findings provide a promising new approach for enhancing tPA thrombolytic therapy.

A number of reports have suggested new strategies for reducing tPA dosage, thereby improving safety at the same time as enhancing plasmin generation or activity. These approaches are based on the following findings: (1) local infusion of plasmin directly into the thrombus does not cause

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**Figure 3.** tPA–rA2 combination therapy reduces hemorrhage volume in rats with ischemic stroke. Rats subjected to focal embolic stroke were treated at 4 hours with saline, standard high-dose tPA (10 mg/kg, H-tPA), or low-dose tPA (2.5 mg/kg, L-tPA) plus rA2 (5 mg/kg). The volumes of intracerebral hemorrhage were quantified by hemoglobin assay at 24 hours after stroke. The data were expressed as mean±SEM, n=10, *P<0.05. Reprinted with permission from Zhu H, Fan X, Yu Z, Liu J, Murata Y, Lu J, Zhao S, Hajjar KA, Lo EH, Wang X. Annexin A2 combined with low-dose tPA improves thrombolytic therapy in a rat model of focal embolic stroke. J Cereb Blood Flow Metab. 2010;30:1137–1146. Copyright 2010, Nature Publishing Group.

**Figure 4.** Neurological scores and infarction volume are improved in rats receiving combination therapy. Two groups of rats were treated intravenously at 4 hours after stroke with either saline or a combination of low-dose tPA (2.5 mg/kg, L-tPA) plus rA2 (5 mg/kg). A, Three days after the onset of stroke, neurological scores of 2 group rats were evaluated. B, Ischemic infarction size on hematoxylin and eosin-stained sections were examined in each group. *P<0.05, n=8 for saline and n=11 for the combination. Reprinted with permission from Zhu H, Fan X, Yu Z, Liu J, Murata Y, Lu J, Zhao S, Hajjar KA, Lo EH, Wang X. Annexin A2 combined with low-dose tPA improves thrombolytic therapy in a rat model of focal embolic stroke. J Cereb Blood Flow Metab. 2010;30:1137–1146. Copyright 2010, Nature Publishing Group.
excessive bleeding within 6-fold greater than the effective thrombolytic dose of tPA used\(^3\); (2) antiplasmin neutralizes circulating plasmin within 1 second of its appearance,\(^3\) whereas tPA has a longer half-life (4 to 5 minutes) and can cross the blood–brain barrier, whether damaged or intact, through low-density lipoprotein receptor-related protein-dependent and -independent mechanisms, further compromising blood–brain barrier integrity and worsening brain damage\(^8,34,35\); (3) tPA can induce plasmin-independent matrix metalloproteinase-9 upregulation and matrix metalloproteinase-9-mediated microglia activation in stroke animal models\(^8,36,37\); and (4) initial experimental evidence suggests that administration of rA2 alone in vivo is not associated with organ-specific or systemic complications.\(^27,28,38\) These findings, together with our preliminary data, suggest that optimization of tPA-mediated fibrinolysis may greatly improve the efficacy and safety of this form of stroke therapy.

Because annexin A2 accelerates the activation of plasmin by complexing with tPA and plasminogen, it is tempting to speculate further that the rA2–tPA combination might generate more plasmin locally at the clot site.\(^39,40\) rA2-associated tPA might generate A2-associated plasmin, which could then interact with fibrin in a thrombus and initiate its degradation, forming FDPs. Alternatively, rA2-associated tPA might interact directly with fibrin within the thrombus, and the tPA might then activate fibrin-bound plasminogen and generate plasmin, which would then dissolve the thrombus, generating FDPs. Both tPA and plasmin would be relatively protected from their circulating inhibitors, plasminogen activator inhibitor-1, and alpha\(_2\)-antiplasmin, respectively.

We speculate that the enhanced thrombolytic efficacy of rA2 plus tPA reflects improved cerebral blood flow. The decrease in side effects associated with this combination therapy may involve a reduction in tPA-associated blood–brain barrier disruption and neurovascular injury. Ultimately, improved blood flow and fewer neurovascular side effects should translate into smaller infarct volume and improved long-term outcome. It is clear that safety issues and all translational aspects of this potential treatment need to be carefully investigated before any future preclinical evaluation.

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Disclosures
None.

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