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Low dose tPA plus annexin A2 combination attenuates tPA delayed treatment-associated hemorrhage and improves recovery in rat embolic focal stroke

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Abstract

We previously have shown that tissue type plasminogen activator (tPA) in combination with its receptor annexin A2 (rA2) protein significantly improved tPA thrombolytic efficacy. In this study we aimed to examine the therapeutic effects of the combination when treated at delayed 4-hour window after stroke compared to standard conventional tPA alone in an embolic focal stroke rat model. We compared effects of intravenous tPA alone (10mg/kg) versus a combination of low-dose tPA (5mg/kg) plus 10 mg/kg rA2. Totally 152 rats were used. Our results showed that: (1) at 24 hours after stroke, the combination slightly reduced brain infarction compared to saline (9.2% reduction), and tPA (7.4% reduction), although the reductions did not reach statistical significance; while the combination significantly reduced (22.2% reduction) the conventional tPA-elevated intracerebral hemorrhagic (ICH) transformation; (2) at 7 days after stroke, the combination significantly attenuated conventional tPA alone-elevated iron deposition at peri-lesion area (68.2% reduction); (3) at 28 days after stroke, the combination significantly improved performance of adhesive tape-removal test, which was accompanied by significantly higher microvessel density at peri-infarct areas compared to conventional tPA alone group. In conclusion, compared to conventional tPA alone, when treated at delayed 4-hour after stroke, the combination

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Conflict of Interest

Yinghua Jinag, Xiang Fan, Zhanyang Yu, Chongjie Chen, Xiaoshu Wang, Eng H. Lo, Xiaochuan Sun, Xiaoying Wang declare that they have no conflict of interest.

Compliance with Ethics Requirements

All institutional and national guidelines for the care and use of laboratory animals were followed.

of low-dose tPA plus rA2 therapy provides a safer profile by lowering risk of ICH transformation and improves neurological function recovery after stroke.

Keywords

annexin A2; tissue plasminogen activator; embolic focal stroke; combination thrombolytic therapy; intracerebral hemorrhagic transformation; long term neurological outcomes

1. Introduction

Intravenous administration of recombinant tPA remains the most commonly used proven beneficial intervention for acute ischemic stroke by stimulating thrombolysis and rescues the ischemic brain via restoring blood flow. However, the short therapeutic time window, ICH transformation, poor thrombolytic perfusion rate, and neurotoxicity comprise major limitations to its use [1–3]. Annexin A2 is a cell-surface protein that can form a triple complex with tPA and plasminogen, which increases the catalytic efficiency of tPA, enabling it to convert plasminogen to plasmin more efficiently in about 60 fold than same amount of tPA alone in vitro [4]. However, clinically giving large amount of tPA alone but without fibrinolytic assembly of the tPA-annexin A2-plasminogen complex formation, makes tPA converting plasminogen to plasmin inefficiently, which may be partially responsible for the shortcomings of tPA reperfusion therapy [4]. Our previous experiments have shown that low-dose tPA combining with its “amplifier” rA2 is more effective than conventional tPA alone when treated within 3 hours after focal embolic stroke in rats [5–7].

The rationale of this study was mainly based on clinical observation that even within the 3-hour time window, intravenous tPA only induce partial or complete reperfusion in about 50% stroke patients, while another 50% patients had no reperfusion (non-responders) at all. These 50% non-responders are not benefited by the tPA reperfusion treatment, but face a higher risk of ICH transformation. Furthermore, the lower reperfusion efficacy and higher hemorrhage risk are also positively associated with treatment time [8]. The purpose of the present study was to ask whether the tPA plus rA2 combination therapy is more efficacious or/and safer compared to the conventional tPA alone, when the treatment time window is delayed at 4 hours after stroke in a embolic focal stroke model of rats.

2. Materials and Methods

2.1 Focal embolic cerebral ischemia in rats

All experiments were performed following an institutionally approved protocol in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male Wistar rats (280–330g) were subjected to focal embolic stroke as we previously described [9]. For this translational study, all stroke therapy academic industry roundtable (STAIR) recommendations and RIGOR guidelines for effective translational research were followed in terms of randomization, blinding and statistical powering [10, 11]. Rats were treated with either saline, or high-dose tPA (10 mg/kg) alone, or combination of low-dose tPA (5 mg/kg) plus rA2 (10mg/kg). Recombinant human tPA (alteplase) was purchased from Genentech. Recombinant annexin A2 (rA2) was produced as previously

described [7]. The rA2 was mixed with tPA for 30 min at 37°C before intravenous infusion. Animals were anesthetized with 1% to 2% isoflurane under spontaneous respiration in a 30% oxygen/70% nitrous oxide mixture. Rats were anesthetized again when treatment were administered. An initial 10% bolus was followed by infusion of the remaining drug over 30 minutes using a syringe pump. Inclusion criteria was set as: (1) stable 50% or less regional cerebral blood flow (rCBF) of pre-ischemic baseline for up to 1 hour after embolization; (2) Modified neurological severity score (mNSS) at 3 hours after stroke is ≥ 8 as we previously established [5]. Totally 152 rats were used.

2.2 Measurements of brain infarct size, intracerebral hemorrhagic volume and mortality at 24 hours after stroke onset

Seven coronal brain sections (2 mm thick) were stained with 2,3,5-triphenyltetrazolium chloride (TTC, Sigma) to quantify infarct size using computer-assisted image analysis. The TTC stained brain sections were used for quantification of intracerebral hemorrhagic volume with spectrophotometric hemoglobin assay as we previously described [7, 9]. Within 24 hours after stroke onset, dead rats were counted for mortality rate.

2.3 Measurements of iron deposition and mortality at 7 days after stroke

It has been reported iron deposition was peaked after 7 days and remaining at a high level thereafter for at least 14 days following ICH in rats [12]. At 7 days after stroke, we detected iron deposition using the Accustain Iron Kit (Sigma-Aldrich) by following manufacture's instructions [13, 14]. For semi-quantitative analysis, on three coronal sections (20- μ m thick, at 2-mm interval) at -0.8, -2.8 and -4.8mm from bregma (the maximal brain infarct area), five consecutive fields ($\times 200$ magnification) along with subcortical peri-infarct area of each section (totally 15 fields per brain) were digitized for determining iron pigment (blue color). Severity of iron deposition was expressed as positive rate of iron deposition presented as the number of iron pigment in observed fields divided by total scanned fields. Within 7 days after stroke onset, dead rats were accounted for mortality rate.

2.4 Assessments of neurological function deficits

Foot fault test and mNSS for motor coordination function, adhesive tape-removal test for sensorimotor neurological deficits were assessed on days 1, 3, 7, 14, 21, and 28 after stroke by following standard methods [15–17].

2.5 Measurements of brain infarction and mortality at 28 days after stroke

H&E staining was used to quantify ischemic brain infarction size at 28 days after stroke as we previously described [17]. Within 28 days after stroke, dead animals were counted for mortality rates.

2.6 Immunohistochemistry and semi-quantification

At 28 days after stroke, immunohistochemistry was performed to determine surrogate biomarkers for neurovascular remodeling in peri-infarct area on three coronal sections at -0.8, -2.8 and -4.8mm from bregma (the maximal brain infarct area) by following standard methods [17]. Primary antibodies against Von Willebrand factor (vWF) (Abbiotec, San

Diego), Vascular endothelial growth factor (VEGF) (Santa Cruz biotechnology, Santa Cruz, CA), and synaptophysin (Chemicon, Temecula, CA) were used. Vessel density (vWF-positive vessels on 3 fields per section in peri-infarct cortex) was quantitated as percentage of vWF positive vessel area; VEGF expression (positive cells area on 3 fields per section in peri-infarct cortex) and synaptophysin expression (positive signals on 3 fields per section in peri-infarct cortex) was quantitated as mean optical density, as we previously described [5].

2.7 Statistical analysis

Infarction and hemorrhage volumes, and immunohistochemistry were analyzed by one-way ANOVA. Mortality rate was analyzed by two-sided Fisher's exact test. Neurobehavioral assessments were analyzed by repeated measures ANOVA followed by post-hoc t-test. The most conservative multiple-test correction was applied using the Bonferroni method. Differences with $P < 0.05$ were considered statistically significant.

3. Results

3.1 Low-dose tPA plus rA2 combination reduces ICH volume at 1 day after stroke

At one day after stroke, infarction volume (% of hemisphere) of the combination group (36.5 ± 6.8) was 9.2% and 7.4% smaller than saline group (40.2 ± 7.3) and tPA alone group (39.4 ± 7.5), respectively. While, ICH volume ($\mu\text{l}/\text{hemisphere}$) was significantly increased in tPA alone group (7.34 ± 1.25) compared to saline control (3.62 ± 1.27), importantly the tPA-elevated ICH was significantly attenuated by the combination treatment (5.71 ± 1.85 , 22% reduction, $P < 0.05$) (Figure 1). No significant difference of one-day mortality was detected between saline (15%, 3/20), tPA alone (25%, 5/20) and combination (10%, 2/20) groups.

3.2 Low-dose tPA plus rA2 combination eliminates iron deposition in peri-lesion area at 7 days after stroke

At 7 days after stroke, positive rate of iron deposition in subcortical peri-infarct areas was significantly increased in tPA alone group ($21.1\% \pm 0.13$) for about 6.4 fold of saline group ($3.3\% \pm 0.04$), but it was significantly reduced by the combination ($6.7\% \pm 0.07$, 68.2% reduction) (Figure 2). Additionally, we found there was a decline of 7-day mortality in the combination group (20%, 2/8) compared to the saline (50%, 4/8) and conventional tPA alone (37.5%, 3/8) groups, but the difference was not statistically significant.

3.3 Low-dose tPA plus rA2 combination improves long-term neurological outcomes

3 neurobehavioral tests were assessed at days 1, 3, 7, 14, 21 and 28 after stroke. No significant differences were detected in two neurobehavioral tests, mNSS score and foot fault tests between the two groups. However, the combination group had a significant better performance in adhesive tape-removal test compared with tPA alone group at both 14 and 28 days, and between the two treatments (Figure 3). No significant differences in brain infarction volume between conventional tPA alone ($23.2 \pm 4.5\%$) and the combination ($22.6 \pm 3.2\%$), and in 28-day mortality between conventional tPA alone (32.4%, 11/34) and the combination (26.5%, 9/34) groups were detected.

3.4 Low dose tPA plus rA2 combination increases cerebrovascular density in the peri-infarct cortex at 28 days after stroke

At 28 days after stroke, we did not detect significant differences in VEGF and synaptophysin expression between tPA alone and the combination groups. While vWF positive area in the combination group was significantly higher (5.1%) compared to the tPA alone group (3.5%, $P<0.05$), demonstrating a significantly higher cerebrovascular density of the combination group at the peri-infarct cortex (Figure 4).

4. Discussion

Our previous studies have shown that the same dose regimen of rA2 plus low dose tPA combination may be a promising combination approach for improving both reperfusion efficacy and reducing ICH at early 3-hour time window [5–7]. The purpose of the present study was to ask whether the low dose tPA plus rA2 combination is still more beneficial than the conventional tPA alone when treated at a delayed 4- hour time window after stroke.

Three sets of experiments were performed. In the first set experiment, we examined ischemic brain infarction and hemorrhagic volumes at 24 hours after stroke. We found a resistance of tPA thrombolytic reperfusion when treated at delayed 4 hours, which was in consistence with previous reports [18]. The thrombolytic reperfusion resistance was reflected by the similar infarct size between saline and conventional tPA alone groups, and limited infarction reduction by the combination (9.2% reduction) compared to saline (Figure 1), however the combination significantly reduced hemorrhagic transformation (22.2% reduction) compared to tPA alone (Figure 1). Clinically, even within the 3-hour time window, intravenous tPA only induces partial or complete reperfusion to about 50% stroke patients. Unfortunately, we are unable to predict who are the tPA reperfusion responders or non-responders before giving tPA clinically. These 50% non-responders are not benefited by the tPA reperfusion treatment, but face a higher risk of complications of tPA thrombolytic therapy-associated ICH transformation [8]. A clinical investigation reported that 3-month mortality was about 60% to ischemic stroke patients with ICH after receiving intravenous tPA administration [19]. Therefore the significantly lower hemorrhagic transformation (22.2%) by the combination treatment is translationally significant.

Brain tissue iron deposition is a common histological biomarker peaked at one week after ICH, and also one of key pathogenic factors that contributes to hemorrhagic brain damage [12, 20]. In the second set experiment, at 7 days after stroke the combination significantly decreased hemorrhagic iron deposition (68.2 % reduction) compared to conventional tPA-alone group (Figure 2). Additionally, there was a lower 7-day mortality in the combination group (25%, 2/8) compared to saline (50%, 4/8) and tPA alone group (37.5%, 3/8). Although the difference of mortality did not reach statistical significance, it might be at least partially due to the smaller sample size examined. The significant reduction of brain iron deposition at sub-acute phase suggested the combination may result in less hemorrhagic brain damage.

In the last set experiment, we aimed to ask whether the significant reduction of ICH by the combination at acute phase may sustain to a significantly improved long-term neurological

function compared to tPA alone. We found there was no significant difference in brain infarct size and mortality between the two treatments at 28 days after stroke. However, the combination group showed a significant better performance in adhesive tape-removal test compared with tPA alone group at both 14 and 28 days, and between the two treatment groups (Figure 3).

Since cerebrovascular remodeling has been thought a key endogenous recovery mechanism [21], in an exploratory experiment, we measured and compared representative surrogate markers for neurovascular remodeling in peri-infarct tissue at 28 days after stroke [5]. We found the combination group had a significantly higher microvessel density at peri-lesion area (Figure 4), suggesting the combination did not dampen, and might slightly augment recovery mechanisms of neurovascular remodeling compared to the standard conventional tPA alone.

We are aware there are several limitations in our present study. Firstly, it would be ideal to have non-treatment saline group enrolled in the long term survival experiment. We did not include saline group was due to that in our 7-day survival experiment, we found about 50% mortality (4/8) within one week in saline control rats, and speculated the 4-week mortality might be unacceptably higher than 50%. (2) Since tPA alone did not, and the combination only reduced 9.2% of brain infarction compared to saline control at 24 hours after stroke, suggested a limited reperfusion might be achieved by the combination but not tPA alone, which might be considered as “non-responders” to those tPA treated rats. (3) However, the tPA alone-elevated hemorrhagic volume at 24 hours after stroke was significantly attenuated (22.2% reduction) by the combination. So we believed without saline control we might still be able to examine at least whether the significant acute hemorrhagic transformation reduction by the combination could sustain to a significantly improved long-term neurological function compared to tPA alone. Nevertheless, it is translationally important that saline control needs to be tested and compared in future preclinical investigations.

Secondly, underlying molecular mechanism for the better neurological function recovery of the combination remains to be further investigated. We might speculate that in addition to the reduction in tPA-associated ICH transformation, the lower tPA dose and formed rA2-tPA complex might limit tPA brain penetration-associated neuronal excitotoxicity [22], and rA2 might bind and neutralize angiostatin (one of tPA-plasminogen converting products)-associated endothelial toxicity [23]. Ultimately, decreased hemorrhagic brain damage and fewer neurovascular side effects of the combination treatment might translate into better vascular remodeling and improved long-term neurological outcome [4].

Thirdly, our focal embolic stroke model of rats used in this study showed tPA thrombolytic resistance when treated at 4 hours after stroke onset, which does not very closely mimic clinically eligible 4.5 hours therapeutic time window [24]. Development of a delayed tPA treatment-responder model by modifying clot preparation for further preclinical evaluation of the combination approach would be translationally important. Lastly, other preclinical pharmacology-related aspects need to be fully evaluated in the future, including dose regimen optimization, testing in animal models carrying stroke comorbidities, and safety related issues [10].

In conclusion, compared to conventional high-dose tPA alone, when treated at delayed 4-hour time point after stroke, the combination of low-dose tPA plus rA2 therapy provides a safer profile by lowering risk of ICH transformation and improves recovery after stroke. Further translational development of this tPA plus rA2 combination stroke therapy may be warranted.

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HIGHLIGHTS

1. Combination of tPA plus rA2 significantly reduces delayed tPA treatment-associated hemorrhagic transformation in stroke rats.
2. The combination significantly reduces iron deposition at 7 days after stroke.
3. The combination significantly improved long term neurological function recovery.

Figure 1.

Brain infarction volume and hemorrhagic transformation at 24 hours after stroke. **A**, Representative images for 2,3,5-triphenyltetrazolium chloride (TTC)-stained ischemic brain infarctions. **B**, Quantification of brain infarction volume. **C**, Intracerebral hemorrhage volumes. Data were expressed as mean $\pm$ SD, *P<0.05, n=17 for saline group, n=15 for tPA alone group, and n=18 for the combination group.

Figure 2.

Iron deposition at 7 days after stroke. **A.** Representative image of iron deposition on rat brain sections treated with conventional tPA alone. Scale bar=50 μ m. **B.** Quantitative data of positive rate of iron deposition. Data are expressed as mean+SD; * P <0.05, n =4 for saline group, n =5 for tPA alone group, and n =6 for the combination group.

Figure 3.

Neurological function deficits. Neurological function deficits were assessed on 1, 3, 7, 14, 21 and 28 days after stroke. **A.** Modified neurological severity. **B.** Foot fault test. **C.** Adhesive removal test. Data were expressed as mean $\pm$ SD, *P<0.05, n=23 for tPA alone group and n=25 for combination group. Mortality of 28 days after stroke was 32.4% (11/34) in conventional tPA alone, 26.5% (9/34) in the combination group, respectively.

Figure 4.

Immunohistochemistry of brain sections. Immunohistochemistry was performed to examine cerebrovascular density, VEGF and synaptophysin expressions in peri-infarct cortex at 28 days after stroke. **A.** Representative immunohistochemistry images for cerebral vessels labeled by endothelial cell maker vWF. **B.** Quantification of cerebrovascular density. **C.** Representative immunohistochemistry images of VEGF expression. **D.** Quantification of VEGF expression. **E.** Representative immunohistochemistry images of synaptophysin

expression. **F.** Quantification of VEGF expression. Data were expressed as mean $\pm$ SD, *P<0.05, n=6 per group was randomly selected.

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