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## Research Report

# Efficacy of recombinant annexin 2 for fibrinolytic therapy in a rat embolic stroke model: A magnetic resonance imaging study

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## ABSTRACT

Efficacy of recombinant annexin 2 (rAN II) in a rat model of embolic stroke was examined using a magnetic resonance imaging (MRI) and histology. The right middle cerebral artery of male Wistar rats was occluded by autologous clots under anesthesia. Four doses of rAN II (0.125, 0.25, 0.5 and 1.0 mg/kg,  $n=10$  for each group) or saline (1 ml/kg,  $n=10$ ) were administered intravenously within 5 min before clot infusion. Serial changes in apparent diffusion coefficient (ADC) and relative blood flow (CBF) were measured with the use of MRI in half of the animals in each group. The remaining half of the animals in each group was evaluated for hemorrhage and final infarct size by histology at 48 h after embolization. At 3 h after embolization, lesion volumes with ADC were abnormality and CBF in the peripheral lesion was improved in groups treated with 0.25, 0.5 and 1.0 mg/kg, but not 0.125 mg/kg, of rAN II in comparison with the saline-treated group ( $P<0.05$ ). Histological analyses were consistent with MRI findings. More importantly, no hemorrhagic transformation was documented in rats treated with 0.125 and 0.25 mg/kg of rAN II, whereas it was observed at higher doses. We concluded that rAN II at 0.25 mg/kg significantly reduced infarct size and improved CBF without hemorrhagic complications. rAN II is a novel compound that has the potential to be a promising fibrinolytic agent to treat embolic stroke.

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## 1. Introduction

Ischemic stroke is the third leading cause of death in industrial nations (Dirnagl et al., 1999; Fisher, 2003). In acute

thromboembolic stroke, one major therapeutic strategy is to recanalize the occluded artery and rescue brain tissues from ischemic damage. The efficacy of fibrinolytic agents, such as the recombinant tissue plasminogen activator (t-PA), has been

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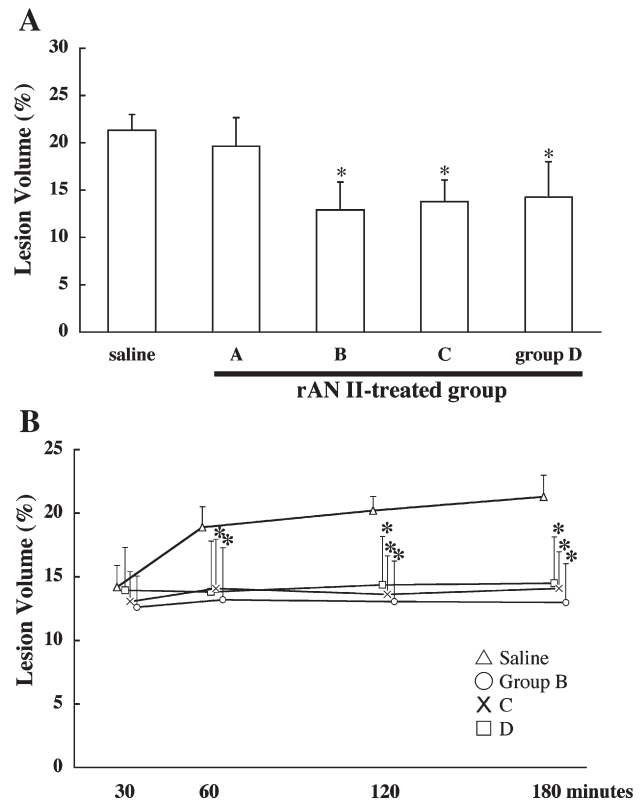
**Table 1 – Physiological parameters**

	Body weight (g)	Blood pressure (mmHg)		pH	pO <sub>2</sub> (mmHg)	pCO <sub>2</sub> (mmHg)
		Before	After			
Saline Group	308±16	120±21	114±12	7.42±0.06	137±26	33±9
Group A	314±28	106±13	118±9	7.44±0.10	129±19	36±5
Group B	303±22	117±15	120±11	7.47±0.11	131±33	32±7
Group C	308±31	116±18	111±16	7.41±0.08	145±21	36±8
Group D	311±22	105±22	116±13	7.39±0.12	135±14	34±4

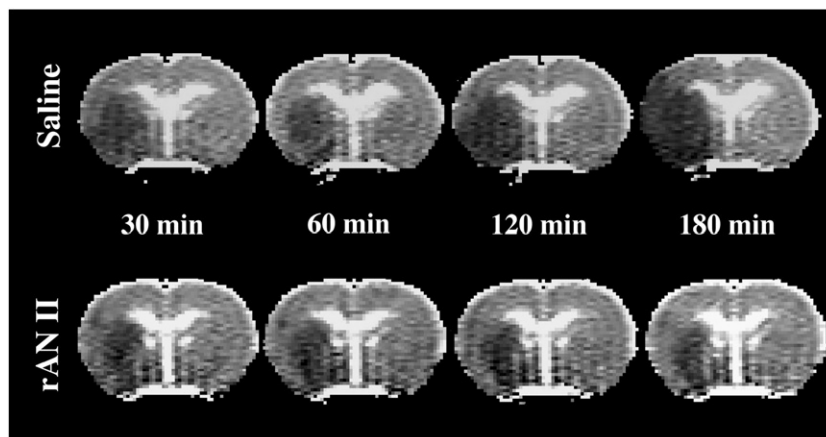
All values are mean±SD. No statistically significant differences between groups.

amply demonstrated to successfully recanalize arterial obstruction and improve clinical outcome (Clark et al., 1999; Hacke et al., 1995, 1998; Lewandowski et al., 1999; National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group (NINDS), 1995). While this treatment is very successful, a significant number of patients who received fibrinolytic therapy showed increased risk of hemorrhage in a time-dependent manner (Dirnagl et al., 1999; Fisher, 2003; Jaillard et al., 1999). Moreover, some experimental studies also exhibited signs of neurotoxicity with t-PA (Kaur et al., 2004; Wang et al., 2003). The search for alternatives to rt-PA aiming to reduce side effects (i.e., hemorrhagic complications) remains an urgent priority.

Annexin 2, a fibrinolytic modulator which binds to t-PA and plasminogen resulting in enhanced plasmin generation, was recently identified in human endothelial cells (Hajjar, 1991, Hajjar et al., 1994). Intravenous administration of recombinant annexin 2 (rAN II) significantly inhibits thrombus formation in vivo without affecting fibrinolytic and coagulation parameters in a rat carotid artery model (Ishii et al., 2001), suggesting rAN II could potentially be used in anti-thrombotic treatment of stroke with minimal side effects.



**Fig. 2 – (A)** The lesion volumes defined by reduced ADC at 180 min after embolization in a rat model of stroke as described in Experimental procedures (saline, n=5; group A, 0.125 mg/kg of recombinant annexin 2, n=5; group B, 0.25 mg/kg, n=5; group C, 0.5 mg/kg, n=5; group D, 1.0 mg/kg, n=5). rAN II-treated groups (groups B, C and D) showed reduction of lesion volume compared with the saline-treated group (\*P<0.05, vs. saline-treated group). **(B)** The temporal profiles of ADC lesion volumes obtained up to 180 min after embolization. At 30 min after embolization, there were no significant differences among groups. However, the lesion volumes did not grow with time in rAN II-treated groups compared to the saline-treated group (\*P<0.05).



**Fig. 1 – ADC maps in representative rats of the saline-treated group A and rAN II group B. The rAN II rat showed smaller lesion that is relatively time independent, whereas saline-treated rat showed a larger lesion, growing in a time-dependent manner.**

In this study, we examined the efficacy of rAN II in treating embolic stroke in a rat model using magnetic resonance imaging (MRI) analysis. Diffusion-weighted imaging (DWI) and perfusion-weighted imaging (PWI) are very sensitive to acute stroke changes (Busch et al., 1997) and they have been widely used to diagnose acute stroke. We used DWI and PWI to non-invasively monitor ischemic evolution and to quantify lesion volumes with and without rAN II treatments in embolic stroke rats. Four different doses of rAN II were studied. MRI data were cross validated with histology. Our results showed that rAN II at an appropriate dose significantly reduced infarct size and improved cerebral blood flow (CBF) without hemorrhagic complications, indicating that rAN II is a potentially promising fibrinolytic agent to treat embolic stroke.

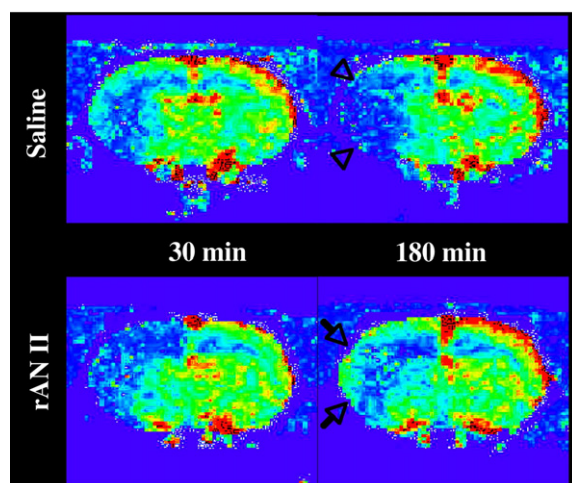
## 2. Results

### 2.1. Physiological parameters

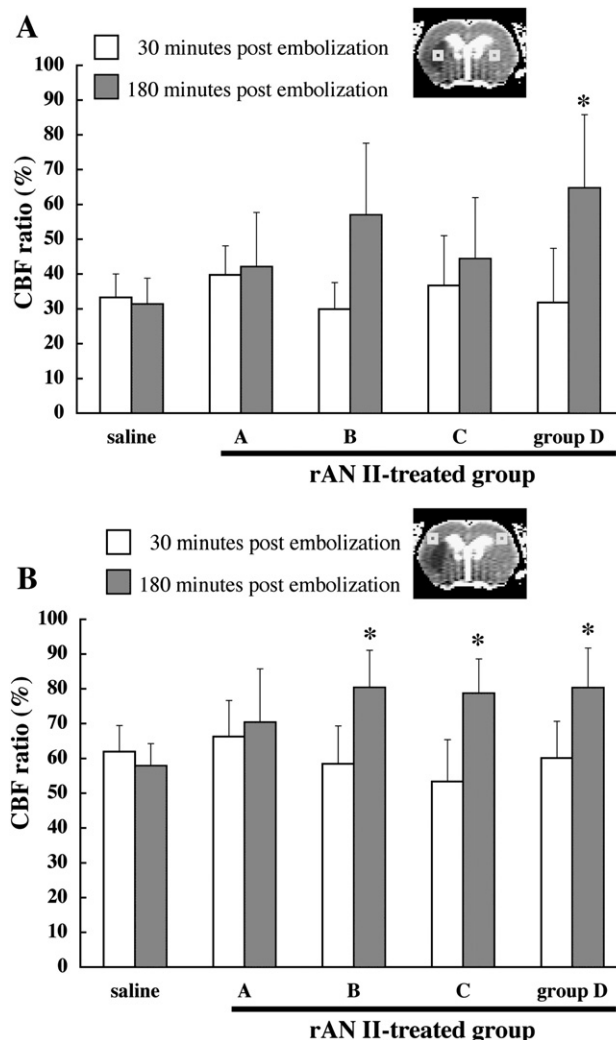
The physiological parameters before and after embolization are summarized in Table 1. All physiological parameters were within the normal range and there were no significant differences between groups.

### 2.2. Reduction in the area with depressed ADC on MRI

Seven rats were excluded from the study because no lesion with reduced ADC was detected 30 min after the clot injection. Fig. 1 shows serial changes in ADC maps after the injection of autologous clots. The lesion volume with a reduced ADC expanded progressively with time in saline-treated rats, while minimal expansion was observed in a moderate to high dose of rAN (>0.25 mg/kg). Fig. 2A illustrates the lesion volume in ADC at 180 min after embolization. In control saline-treated rats, the lesion volume was  $21 \pm 2\%$  of the ipsilateral hemisphere. In contrast, it was significantly reduced in the rats



**Fig. 3** – The temporal profile of rCBF maps in representative rats of the saline-treated group and rAN II group B. rAN II-treated rat showed partial improvement at 180 min (open arrows), whereas the saline-treated rat showed no improvement (open arrowheads).



**Fig. 4** – The CBF ratios of the ipsilateral to contralateral hemisphere at 30 and 180 min after embolization in each group (saline,  $n=5$ ; group A, 0.125 mg/kg of rAN II,  $n=5$ ; group B, 0.25 mg/kg,  $n=5$ ; group C, 0.5 mg/kg,  $n=5$ ; group D, 1.0 mg/kg,  $n=5$ ). (A) In the ischemic core region, groups B and D showed statistical significant improvement. (B) In the penumbral region, rAN II-treated groups (groups B, C and D) showed significant improvement in CBF ratios, compared to the CBF ratios at 30 min (\* $P<0.05$ ).

treated with rAN II ( $13 \pm 3\%$  for group B;  $14 \pm 2\%$  for group C;  $14 \pm 4\%$  for group D). Fig. 2B shows the temporal profiles of lesion volume. At 30 min after embolization, no significant differences were observed among all groups, including the control saline-treated group ( $14 \pm 2\%$  of saline group;  $13 \pm 2\%$  of group B;  $13 \pm 2\%$  of group C; and  $14 \pm 3\%$  of group D). However, the lesion volume in the saline-treated group expanded progressively in a time-dependent manner, suggesting that rAN II attenuated future deterioration in ischemic injury.

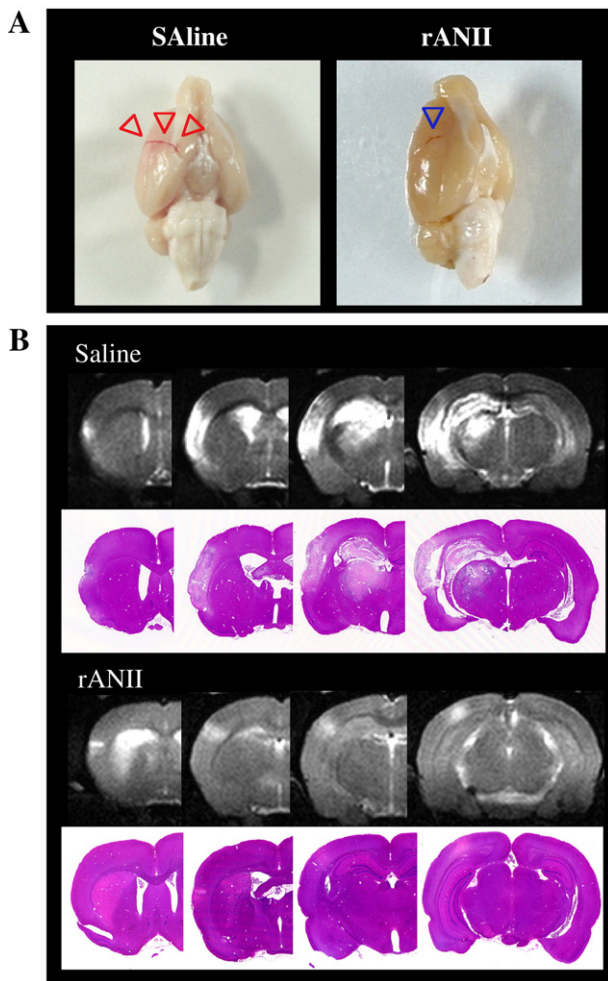
### 2.3. rCBF measurement with the use of PWI

We evaluated CBF ratio, another index of brain ischemia. Fig. 3 shows the rCBF maps and Fig. 4 compares the ipsilateral:

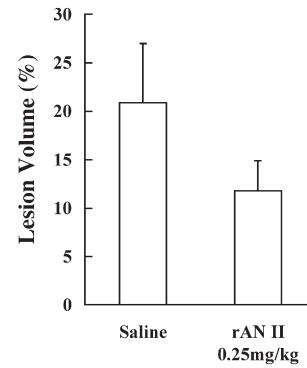
contralateral CBF ratio at 30 min and 180 min after stroke. Comparable CBF reduction was observed among different groups in both ischemic core and non-ischemic core region, as demonstrated by the reduced CBF ratio (Fig. 4). At 30 min after embolization, rCBF ratio decreased to ~40% and ~60%, in ischemic core and non-ischemic core region, respectively. No significant differences in CBF ratio were detected among all groups at 30 min. In contrast, significant differences became clear at 180 min among groups. No improvement in rCBF was observed in the saline-treated group and the low dose (0.125 mg/kg) rAN II-treated group. However, significant improvement was achieved in group B and D, especially in peripheral region.

#### 2.4. Histopathological findings

The occluding locations of MCA were confirmed with macrographical examinations of the transcordially fixed brain (Fig. 5A). Clots were observed over ~1 cm from proximal to



**Fig. 5 – (A) Macroscopic examination of the brain showed the occlusion of right MCA with a relatively large clot (red arrowheads) in saline-treated rats. In contrast, in rAN II-treated rats, the size of the occluded clot (blue arrowhead) was relatively small. (B) T2-weighted magnetic resonance images and corresponding brain slices of two representative rats in each group.**



**Fig. 6 – Infarct volumes by histology at 48 h for saline-treated group (group A) and 0.25 mg/kg rAN II-treated group (group B). There was significant difference between 0.25 mg/kg rAN II-treated rats (12 ± 3%) and saline-treated rats (21 ± 6%).**

distal MCA in all saline-treated rats, while small clots were seen only at distal MCA in rAN II-treated rats.

The final infarct size and hemorrhagic complication were confirmed in subgroups at 48 h post-operation. Fig. 5B demonstrates representative T2-weighted images and macroscopic findings of saline-treated rat and group B rAN II-treated (0.25 mg/kg) rat at 48 h after embolization. Intracranial hemorrhage was identified in 3 (60%) and 2 (40%) of the rats treated with 1.0 mg/kg and 0.5 mg/kg of rAN II, respectively, and these 5 rats died at 48 h after embolization. On the other hand, no hemorrhagic transformations were documented in rats treated with a lower dose of rAN II (<0.25 mg) or with control saline. To evaluate the ischemic damages, histological findings were compared between 0.25 mg/kg rAN II-treated and saline-treated rats (Fig. 6). There was a significant difference in the lesion volume between 0.25 mg/kg rAN II rats and saline-treated rats (0.25 mg/kg rAN II: 12 ± 3%, saline: 21 ± 6%), which were consistent with MR findings.

#### 2.5. Fibrinolytic parameters and coagulation parameters

The data of fibrinolytic and coagulation parameters are summarized in Table 2. There were no significant differences in any of these parameters between rAN II-treated and saline-treated rats.

**Table 2 – Systemic parameters of fibrinolysis and coagulation**

	PT (s)	APTT (s)	FDP (μg/ml)	D-dimer (μg/ml)	PIC (μg/ml)
Saline	13.43 ± 0.84	36.7 ± 7.72	<5	<0.5	<0.3
0.125 mg/kg	13.40 ± 5.31	49.13 ± 13.21	<5	<0.5	<0.3
0.25 mg/kg	14.05 ± 0.35	52.83 ± 10.13	<5	<0.5	<0.3
0.5 mg/kg	13.67 ± 0.90	34.7 ± 7.47	<5	<0.5	<0.3
1.0 mg/kg	13.83 ± 0.61	40.93 ± 7.07	<5	<0.5	<0.3

All values are mean ± SD. No statistically significant differences between groups.

PT, prothrombin time; APTT, active partial thromboplastin time; FDP, fibrin degenerative products; PIC, alpha 2-plasmin inhibitor-plasmin complex.

### 3. Discussion

This study demonstrates that intravenous administration of rAN II at an appropriate dose significantly reduces infarct size and improves CBF without hemorrhagic complications, as assessed by MRI and histological analysis. These data suggest that rAN II partially or completely prevents embolic clots formation and results reduced cerebral infarct volumes.

At the 0.125 mg/kg dose (group A), rAN II did not reduce ADC lesion volume compared to saline-treated group. On the other hand, at the dose of 0.25 mg/kg or higher (groups B, C & D), ADC lesion volume was markedly reduced. These results were confirmed by histological examination at 48 h after embolization. In this study, rAN II was injected at almost the same time of clot injection, and ADC lesion volume at 30 min showed no significant differences among the groups. If rAN II was to directly affect clots soon after embolization, smaller lesions should have been detected by DWI and PWI at 30 min because smaller clots can migrate into distal vascular tree. Therefore, it was indicated that clot size was unlikely affected by rAN II at least in the initial phase. On the other hand, PWI showed CBF improvement at 180min after stroke induction among groups B, C and D. Therefore, we speculated that sufficient dose of rAN II dissolved the blood clots and improved reperfusion between 30 and 180 min after stroke, resulted in reducing infarct volume, rather than preventing secondary thrombi formation. Future studies will test rAN II administration at different time points after stroke to evaluate its clinical potential.

Annexin 2, a phospholipid-binding protein found on cell surface, has been identified as a co-receptor for both plasminogen and t-PA (Hajjar and Hamel, 1990, 1991, 1994). We have previously shown that annexin 2 is attached to endothelial cells in a calcium-dependent manner and fibrinolytic activity disappears in the absence of calcium (Ishii et al., 2001). rAN II restored impaired fibrinolytic activity and sustained carotid flow when rAN II was present on the endothelium. Deletion of the C-terminal 224 amino acid results in a lost fibrinolytic activity of annexin 2 (Ishii et al., 2001), suggesting that C-terminal 224 amino acids in annexin 2 plays important in phospholipid binding to plasminogen. Moreover, rAN II inhibits the formation of thrombus and overexpressed annexin 2 protein on intima of rat carotid artery can be detected by fluorescent immunochemistry. Taken together, annexin 2 upregulates plasmin generation via boost of t-PA and plasminogen on endothelial cells, resolving blood clots and preventing secondary thrombi formation. Secondary thrombi formation is often observed in clinical embolic infarction.

Interestingly, there were no significant differences in ADC lesion volume at 180 min after stroke among groups B, C and D. These results are consistent with our previous observation that rAN II-promoted plasmin generation may have been saturated at higher concentrations (Ishii et al., 2001).

Currently, t-PA is the only agent approved by FDA for stroke treatment (NINDS, 1995). Although proper use of t-PA is beneficial in salvaging ischemic brain tissue (Busch et al., 1998; Clark et al., 1999; Hacke et al., 1995, 1998; Lewandowski et al., 1999; NINDS, 1995; Shen et al., 2004a,b; Hara et al., 1990),

improper use of t-PA could induce serious brain damages (Fiorelli et al., 1999; Kano et al., 2000; Larrue et al., 2001; Wardlaw et al., 2003). t-PA-induced brain damages are generally categorized into neurotoxicity and blood-brain barrier disruption. Neurotoxicity induced by t-PA has been thought to be mediated by N-methyl-D-aspartate excitotoxicity (Fernandez-Monreal et al., 2004) and microglial inflammation (Siao and Tsirka, 2002). For microglial activation, t-PA is expected to require annexin 2 as its mediator. Thus, rAN II for fibrinolytic therapy in cerebral ischemia may also cause neurotoxicity. Blood-brain barrier disruption could cause hemorrhagic transformation, resulting in life-threatening events (Dijkhuizen et al., 2001; Kahles et al., 2005). The exact mechanisms of t-PA-induced blood brain-barrier damage following cerebral ischemia remain poorly understood. Some authors speculate t-PA upregulates brain matrix metalloproteinase-9 (MMP-9) levels in stroke (Wang et al., 2003; Tsuji et al., 2005), resulting in increased vascular permeability. To the contrary, others found that a plasminogen and MMP-9 had an independent mechanism of blood-brain barrier damage through t-PA (Yepes et al., 2003).

Our results demonstrated that rAN II showed effective fibrinolysis without hemorrhagic complication in appropriate dose (0.25 mg/kg), while high dose of rAN II (0.5–1.0mg/kg) caused hemorrhagic transformation. High dose of rAN II has similar hemorrhagic transformation rate as t-PA (Kano et al., 2000; Tejima et al., 2001). Recent report demonstrated annexin 2 also increases MMP-9 level by facilitating the interaction between t-PA and plasminogen (Hwang et al., 2006). Therefore, our results suggest that MMP-9 is upregulated by excessive rAN II, increasing hemorrhagic transformation.

We found that annexin 2 did not induce abnormalities in systemic parameters of fibrinolysis and coagulation. In contrast, t-PA has been shown to induce changes hemostatic parameters such as active partial thromboplastin time (Kase et al., 1990; Ho and Wang, 1990). These data support the notion that the majority of exogenous rAN II may bind to plasma membranes soon after injection due to its high affinity for phospholipids, resulting in low plasma concentration below detection limits. The unique features of annexin 2 may prove to have reduced clinical complications compared to t-PA.

DWI and PWI can monitor stroke evolution during the acute phase in a totally non-invasive manner. Brain tissues with perfusion deficits below a critical threshold (Hossmann, 1994) experience metabolic energy failure, membrane depolarization and subsequent cellular swelling. These changes precipitate a reduction in the apparent diffusion coefficient (ADC) of brain water and are manifested as hyperintense regions on DWI (Busch et al., 1997). During the acute phase, the DWI abnormality is initially smaller relative to the area of perfusion deficit. As ischemia evolves, most of this DWI abnormality expands and eventually coincides with the abnormal PWI area if the stroke is left untreated (Meng et al., 2004; Shen et al., 2004a,b). The difference in the abnormal region defined by the PWI and DWI in acute stroke is commonly referred to as the “perfusion-diffusion” mismatch. It has been suggested that the “perfusion-diffusion” mismatch is potentially salvageable and approximates the ischemic penumbra (Astrup et al., 1981). This methodology enabled us to follow lesion development and to make more careful assessments of brain infarction

without sacrificing animals. Moreover, the combination of DWI and PWI could also offer the possibilities to distinguish primary ischemic damages from the secondary damages such as secondary thrombi formation.

In this study, we defined the ischemic lesion on the ADC map by using the threshold of 80% of the contralateral hemisphere. ADC values vary in different brain regions and the ideal experiment would be to perform a pre-stroke scan and comparison be made on the same pixel pre-stroke. The second ideal experiment would be to compare with homologous pixels in the normal hemisphere. However, both are relatively difficult and suffer from co-registration accuracy. In rats, the brain has relatively homogeneous ADC consisting primarily gray matter with little white matter, in contrast to human brain. A single ADC serves as a good approximation and has been widely used to study stroke (Busch et al., 1998; Meng et al., 2004). Actually, in our preliminary studies, regional ADC values were figured out in the normal rat brain;  $(0.71 \pm 0.015) \times 10^{-3}$  mm<sup>2</sup>/s in gray matter and  $(0.68 \pm 0.036) \times 10^{-3}$  mm<sup>2</sup>/s in white matter. It was  $(0.33 \pm .089) \times 10^{-3}$  mm<sup>2</sup>/s in the ischemic core in this study, corresponding to ~80% of normal ADC value. Thus, the threshold was indeed justified and retrospectively validated.

In conclusion, we demonstrate that rAN II at an appropriate dose significantly reduces infarct volume without hemorrhagic complications. We conclude that rAN II is a promising fibrinolytic agent. A combination therapy of rt-PA and rAN II could further enhance fibrinolytic effects while minimizing hemorrhagic complications. Further investigation with treatment at different time points after embolization is required for better modeling for the clinical implication.

## 4. Experimental procedures

### 4.1. Animal preparation and physiological monitoring

The animal experiments were performed in accordance with our institutional guidelines for animal research. Male Wistar rats weighing 250–350 g were anesthetized with 1.5% isoflurane under intubation and mechanically ventilated. Body temperature was maintained at 37–38 °C throughout the experiment, using a water heating system. The left femoral artery and vein were cannulated with a PE-50 catheter for continuous monitoring of blood pressure and drug injection, respectively. Arterial blood was collected for measurement of blood gases (pH, pO<sub>2</sub>, pCO<sub>2</sub>). Blood collection was performed only one time (30 min after clot injection) in order to avoid the physiological changes due to excessive blood draw. The inspired oxygen (FiO<sub>2</sub>) and expired carbon dioxide (EtCO<sub>2</sub>) concentrations were monitored continuously, and tidal volume was adjusted to produce EtCO<sub>2</sub> of 35–45 mmHg throughout the experiments.

### 4.2. Preparation of the embolus

The method used to prepare the embolus was adapted and modified from previous reports (Busch et al., 1998; Overgaard et al., 1992; Tanaka et al., 2003). Autologous blood was collected 24 h before surgery and retained for 2 h at 20 °C, and for 22 h at 4 °C. The spontaneously formed blood clot was then passed

through a PE-10 catheter (inner diameter 0.28 mm) and cut into small cylindrical segments with a length of 1.5 mm each. To minimize spontaneous lysis, we selected ten of the white clots as the embolic material (Kirchhof et al., 2002), which were transferred into a PE-50 catheter filled with saline for embolization.

### 4.3. Animal surgery

After surgical preparation of the right carotid artery, the pterygopalatine and occipital arteries were ligated. The PE-50 catheter, containing the blood clots, was inserted into the external carotid artery, with its tip located close to the carotid bifurcation. The clots were then injected over a period of 1 min, during which the common carotid artery was temporarily occluded. Heparin was not provided to any animal during the experiment.

### 4.4. Recombinant annexin 2 administration

The method used to prepare rAN II was previously described in details (Ishii et al., 2001). Animals were treated with various doses of rAN II or saline in a blinded manner and investigated after embolization in rAN II-treated group, rAN II was administered intravenously less than 5 min before the induction of embolic stroke. rAN II were used in this experiment at 0.125 mg/kg (group A, n=10), 0.25 mg/kg (group B, n=10), 0.5 mg/kg (group C, n=10) and 1.0 mg/kg (group D, n=10). In the control group, 1.0 ml saline was used in an identical fashion (n=10).

### 4.5. MRI measurement

MRI studies were performed using a 4.7-T superconducting magnet with a 33-cm horizontal bore magnet and a 65-mT/m maximum gradient capability (Unity INOVA, Varian, Palo Alto, CA, USA). A 20-mm diameter surface coil was tuned to 200 MHz for MRI signal reception. Following the surgical procedure, MRI experiments were performed in half of the animals (n=5 in each groups). The remaining animals allowed recovering immediately after stroke induction and treatments for histological examinations at 48 h. For MRI examination, the animal was fixed firmly on the surface coil using a custom-made head sets. DWI was performed up to 180 min after embolization every 30 min, using spin-echo sequence with parameters of 1500/80 ms (TR/TE), a matrix of 128×64, a field of view of 35×35 mm, a section thickness of 2 mm without an intersection gap, a single excitation (no signal averaging) and a total of 7 slices, which started from the pole of cerebrum. The diffusion gradients were applied along the three orthogonal directions (x-, y- and z-axes) to obtain quantitative ADC values with the gradient factor b of 0 and 1200 s/mm<sup>2</sup> (Miyasaka et al., 2000). DWI with the total acquisition time of 7 min repeated consecutively for 180 min after embolization. Dynamic contrast enhanced PWI were performed at 30 min and 180 min after embolization using fast low angle shot sequence. A 2-mm single slice was selected where the largest lesion was observed in ADC map at 30 min after embolization. PWI was acquired with parameters of 10/5 ms (TR/TE), a field of view of 35×35 mm and a matrix of 64×64. A total of 80 scans were obtained, resulting in a total scan time of

approximately 1 min. At acquisition of the 17th image, 0.2 mg/kg of gadopentetate dimeglumine followed by 0.5 ml saline was injected via left femoral vein.

#### 4.6. MRI data analysis

In this study, MRI and histological data analyses were performed by the investigators who were blinded to the treatment assignment. MRI data was transmitted to a PC and analyzed using image-processing software MRVision (MRVision Corp., Menlo Park, CA, USA). ADC maps were generated on a pixel-by-pixel basis using the equation  $ADC = -\ln(S_0/S_1)/(b_1 - b_0)$ , where  $S_0$  and  $S_1$  are the signals of the two DWI that represent the average of 3 orthogonal planes, and  $b_0$  and  $b_1 = 0$  and  $1200 \text{ s/mm}^2$ , respectively. For PWI analysis, the method to calculate rCBF was adapted and modified from previous report (Heiland et al., 1999).  $\Delta R_2^*$  as a change in relaxation rate was calculated using the equation:  $\Delta R_2^*(t) = -\ln[S_i(t)/S_0]/TE$ , where  $S_i(t)$  is the signal intensity at the time point  $t$ , and  $S_0$  is the averaged signal intensity at the time point before injection. A gamma variate fit was used on a pixel-by-pixel basis before calculation of the parameters. Relative cerebral blood volume was calculated as the integral under the fitted transit curve of  $\Delta R_2^*(t)$  ( $\int \Delta R_2^*(t) dt$ ) and normalized first moment of the concentration-time curve was determined as the mean values of the integral under the fitted transit curve of  $\Delta R_2^*$ , up to the peak of the curve ( $\int t^* \Delta R_2^*(t) dt / \int \Delta R_2^*(t) dt$ ). Then, rCBF was estimated by the equation: rCBF = relative cerebral blood volume / normalized first moment. The arterial input function was not applied to calculate perfusion parameters because of the difficulties to identify big artery in this imaging resolution.

The areas, where ADC values decreased to less than 80% of the contralateral hemisphere (Busch et al., 1998), were defined as ischemic lesions. The lesion volume was calculated as a percentage of the volume of contralateral hemisphere (Swanson et al., 1990).

Regions of interest (ROIs), a  $10 \times 10$  pixel area in the center of the ischemic lesion, and another in the peri-ischemic lesion, which were determined from the ADC map at 30 min after embolization, were chosen on the rCBF map. Other ROIs in the homologous region of the contralateral hemisphere were also chosen to calculate CBF ratio. A CBF ratio was determined by dividing the rCBF of ipsilateral hemisphere by that of the contralateral hemisphere.

#### 4.7. Histological investigation

In order to observe the remaining blood clot, half of the animals in each group ( $n=5$ ) were transcatheterially perfused with heparinized saline and 10% buffered formalin under deep anesthesia soon after MRI examinations (180 min after embolization). The others ( $n=5$  in each group) were observed for 48 h post-operation before trans-cardiac perfusion in order to evaluate the final infarct size and risk for hemorrhagic complication. The latter subgroups did not have 180 min MRI investigation to minimize the effect of anesthesia but had conventional T2-weighted image at 48 h after embolization. The brain was immersed in formalin solution, after which the brain was cut into 2-mm-thick coronal blocks, for a total of 7 blocks per animal. Then the brain tissue was processed,

embedded and 6- $\mu\text{m}$ -thick paraffin sections from each block were cut and stained with hematoxylin-eosin (H&E) for the evaluation of ischemic tissue damage.

The infarct volume was measured using NIH image. Each section was evaluated at  $2.5\times$  magnification. The area of infarction and the area of both hemispheres were calculated on H&E-stained sections by tracing the areas on the computer screen, and the volumes were determined by integrating the appropriate area with the slice thickness. To reduce errors associated with processing of tissue for histological analysis, the infarct volume was presented as the percentage of the contralateral hemisphere with edema correction (Swanson et al., 1990).

#### 4.8. Fibrinolytic parameters and coagulation parameters

To evaluate the effect of rAN II in systemic parameters of fibrinolysis and coagulation, blood samples were collected at the end of experiment for measuring the followings parameters: prothrombin time using the Quick-one step method (SRL Inc., Tokyo, Japan), active partial thromboplastin time using the Proctor method (SRL Inc., Tokyo, Japan), fibrin degeneration products using the agglutination method, D-dimer and alpha 2-plasmin inhibitor-plasmin complex using Latex Photometric Immunoassay.

#### 4.9. Statistical analysis

All data are presented as mean  $\pm$  SD. A one-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference (PLSD) was used for group comparison of physiological parameters, fibrinolytic parameters, coagulation parameters and MRI-derived lesion volume. Repeated measure ANOVA followed by Fisher's PLSD was used to compare the MRI-derived lesion volume between saline-treated group and rAN II-treated groups. Paired t-test was used to compare the CBF ratio between two measurement time points in each group and to compare the final infarct volume between rAN II-treated groups and saline-treated group. Differences with  $P$  values of less than 0.05 were considered statistically significant.

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