Quantitative Imaging of Cerebral Thromboemboli In Vivo
The Effects of Tissue-Type Plasminogen Activator

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Background and Purpose—Quantitative imaging for the noninvasive assessment of thrombolysis is needed to advance basic and clinical thrombosis-related research and tailor tissue-type plasminogen activator (tPA) treatment for stroke patients. We quantified the evolution of cerebral thromboemboli using fibrin-targeted glycol chitosan–coated gold nanoparticles and microcomputed tomography, with/without tPA therapy.

Methods—We injected thrombi into the distal internal carotid artery in mice (n=50). Fifty-five minutes later, we injected fibrin-targeted glycol chitosan–coated gold nanoparticles, and 5 minutes after that, we treated animals with tPA or not (25 mg/kg). We acquired serial microcomputed tomography images for 24 hours posttreatment.

Results—Thrombus burden at baseline was 784×10³±59×10³ μm² for the tPA group (n=42) and 655×10³±103×10³ μm² for the saline group (n=8; P=0.37). Thrombus shrinkage began at 0.5 to 1 hour after tPA therapy, with a maximum initial rate of change at 460±957 μm²/min. The rate of change lowered to ≈1% of the initial in hours 1 to 2, followed by ≈29% and ≈1% in hours 2 to 3 and 3 to 24, respectively. Thus, 85% of total thrombolysis over 24 hours (≈500 μm²), equivalent to 64% of the baseline thrombus burden) occurred within the first 3 hours of treatment. Thrombus burden at 24 hours could be predicted at around 1.5 to 2 hours. Saline treatment was not associated with significant changes in the thrombus burden. Infarct size was smaller in the tPA group versus saline group (18.1±2.3 versus 45.8±3.3 mm²; P<0.01). Infarct size correlated to final thrombus burden (r=0.71; P<0.01). Time to thrombolysis, completeness of thrombolysis, and tPA therapy were independent predictors of infarct size.

Conclusions—Thromboembolic burden and the efficacy of tPA therapy can be assessed serially, noninvasively, and quantitatively using high-resolution microcomputed tomography and a fibrin-binding nanoparticle imaging agent.

(Stroke. 2017;48:1376-1385. DOI: 10.1161/STROKEAHA.117.016511.)

Key Words: direct thrombus imaging ■ gold nanoparticles ■ microCT ■ thrombus evolution ■ tissue-type plasminogen activator

Tissue-type plasminogen activator (tPA) is the only Food and Drug Administration–approved drug for treating acute ischemic stroke.1 Patients treated with intravenous tPA within 4.5 hours of stroke onset are more likely to have favorable outcomes than those who do not receive tPA treatment.2 Nevertheless, ≈60% of stroke patients who receive tPA either die or become disabled, despite treatment.3 There is a need for a prompt and precise strategy to identify nonresponders to intravenous tPA1 and, thus, customize recanalization therapy, including endovascular therapy. However, how we should define tPA nonresponders still remains unclear.4 tPA resistance, which partly depends on thrombus characteristics4–7 including age, burden, and composition, is observed in ≈50% of patients. In addition, recanalization is frequently followed by reocclusion or secondary embolization and subsequent neurological deterioration.5,6,8 Other studies on intravenous tPA therapy showed that ≈40% of successful arterial recanalization was not associated with early clinical improvement, whereas about one third of patients without early improvement had good outcomes at 3 months.9 Thus, it was recently suggested that important factors for identifying tPA failures should include thrombus location, burden,
and composition, as well as arterial recanalization and clinical improvement. With few exceptions, we are currently limited to characterizing cerebral thromboemboli in clinic by means of negative contrast.

To overcome many of the pitfalls that current thrombus imaging techniques have (Introduction in the online-only Data Supplement), we have recently developed a direct thrombus imaging technique using computed tomography (CT) and fibrin-targeted glycol chitosan–coated gold nanoparticles (AuNPs). We have noted that since tPA was first introduced in 1996, no study—to our knowledge—has quantitatively assessed a detailed spatial and temporal evolution of cerebral thromboemboli before, during, and after tPA therapy. In the present study, using a mouse model of embolic stroke, we demonstrate that high-resolution in vivo micro-CT (mCT)–based direct thrombus imaging using fibrin-targeted glycol chitosan–coated AuNP enables us to study the tPA-mediated dynamic evolution of cerebral thromboemboli in a quantitative manner.

**Materials and Methods**

This study was approved by the Institutional Animal Care and Use Committee at Dongguk University Ilsan Hospital, and all experiments were conducted in accordance with the institutional guidelines on humane care and use of laboratory animals. Embolic stroke was induced (n=50 mice), as previously reported by injecting preformed fluorescently marked clots into the middle cerebral artery–anterior cerebral artery bifurcation area using a catheter placed in the distal common carotid artery after insertion through a hole made in the external carotid artery. One hour later, mCT images were acquired 5 minutes after intravenous administration of 300 μL fibrin-targeted glycol chitosan–coated AuNPs that we synthesized. These animals were then treated intravenously with either 1 mg/mL tPA (25 mg/kg, 600 μL) or saline (600 μL) bolus injection (10%), which was followed by continuous infusion (90%) for 30 minutes. Serial mCT images were acquired immediately after the bolus (designated as 0 hour) and at 0.5, 1, 1.5, 2, 3, and 24 hours after the bolus/start of the infusion. Once the scheduled imaging sessions were completed, brains were harvested and immediately imaged using a near-infrared fluorescence (NIRF) imager to use the area of thrombus-related fluorescent signal as a reference value to be compared with the area of hyperdense marked thrombus on mCT. Then, 2,3,5-triphenyl-tetrazolium chloride staining was performed to delineate infarcted areas. Finally, quantitative image analyses and infarct mapping were performed. More details are available in the Methods (including Figure I) in the online-only Data Supplement.

**Results**

**Total Thrombus Burden and Thrombolysis**

The total thrombus burden prior to therapy was 784±10^3±59×10^3 μm^2 for the tPA group (n=42) and 655±10^3±105×10^3 μm^2 for the saline group (n=8; P=0.37). The total thrombus burden after treatment was 287±10^3±54×10^3 μm^2 for tPA (≈37% of baseline) and 625±10^3±89×10^3 μm^2 for saline (≈95% of baseline; P<0.01).

**Significant Thrombus Burden Reduction Began 0.5 to 1 Hour After the Start of tPA Therapy, With the Largest Thrombolytic Effect Seen Initially and Attenuated Out to 24 Hours**

Thrombolytic therapy was initiated 1 hour after thrombus deposition, but the thrombus area of the entire brain did not significantly change until 30 minutes after tPA (Figure 1). Specifically, the thrombus area did not significantly reduce either after tPA loading or during the subsequent 30-minute infusion (both corrected P [P corr]>0.05). One hour after tPA administration began, the thrombus area became significantly smaller than baseline (P corr<0.01). This indicates a lag time between the start of tPA treatment and the initial thrombolytic effect, which appeared somewhere between the 0.5- and 1-hour time points (Figures 1 and 2). The thrombus reduction rate between those time points was 4603±957 μm^2/min, as calculated by \((\text{area}_{0.5\text{h}}-\text{area}_{1\text{h}})/\text{time}\) and was the highest rate of change throughout the 24-hour measurement period (Figure 2).

During the 2 hours after this initial high-change phase, thrombus area reduction continued but at a lower rate of change. About 61% of the first thrombolytic effect (i.e., 39% lower than the initial thrombus reduction rate) occurred 1 to 2 hours after the start of tPA and ≈29% (i.e., 71% lower than the initial rate) during hours 2 to 3.

Thrombolysis continued at later times, though much weaker. More specifically, thrombus area reduction sustained out to 24 hours, but the rate of change was as low as 57±20 μm^2/min during hours 3 to 24, which is only ≈1% of the strong primary thrombolytic effect observed during 0.5- to 1-hour period. During hours 3 to 24, however, a considerable amount of thrombus was dissolved, so that the thrombus area was significantly reduced from 359±10^3±56×10^3 μm^2 to 287±10^3±54×10^3 μm^2 (P corr=0.02). In other words, ≈5000×10^3 μm^2 thrombus (≈64% of the baseline burden) that was dissolved in the 24 hours after tPA therapy, ≈85% was cleared during the first 3 hours, while the other 15% was cleared during the next 21 hours.

Unlike tPA treatment, saline treatment did not show significant change in the thrombus area (Figure 1; Figure II and Results in the online-only Data Supplement).

**The Final Thrombus Burden 24 Hours After the Start of tPA Therapy Could Be Predicted by mCT-Measured Thrombolytic Effect at 1.5 to 2 Hours and Residual Thrombus Burden at 2 Hours**

We observed no significant correlation between the initial thrombolytic effect and any subsequent thrombus reduction rates, that is, at hours 1 to 1.5, 1.5 to 2, 2 to 3, or 3 to 24 hours (all P>0.05; data not shown), thereby indicating that the earliest evidence of significant thrombolysis did not predict continued thrombolysis at later time points. However, there was a modest correlation between the thrombus reduction rates at hours 1.5 to 2 versus those at hours 3 to 24 (r=0.68; P<0.01; Figure IIIA in the online-only Data Supplement). This relationship indicates that relatively early, although not the earliest, evidence of thrombolysis may predict continued thrombolysis later on. In addition, the linear associations between the thrombus areas at 24 hours versus prior time points (i.e, baseline to 3 hours) tended to strengthen gradually over time and plateau at 2 hours (Figure IIIB in the online-only Data Supplement). As shown in Figure IIIC and IIID in the online-only Data Supplement, mCT-measured thrombus burden =2 hours after the start of tPA therapy strongly predicts thrombus burden at both 3 hours (r=0.92; P<0.01) and 24 hours (r=0.89; P<0.01).
The circle of Willis (COW) thrombus burden prior to therapy was 535×10^3±43×10^3 μm^2 for the tPA group and 508×10^3±105×10^3 μm^2 for the saline group (P=0.80), that is, ≈68% and 77%, respectively, of the total thrombus burden. As expected, tPA-mediated (versus saline-associated) thrombus evolution in the COW was similar to that in the entire brain (Results in the online-only Data...
However, during the first 3 hours after saline loading and infusion, there was an 8.5±2.7% increase in COW thrombus area, as compared with pretreatment baseline (P corr<0.01; Figure IVA in the online-only Data Supplement). In addition, tPA showed a largely homogeneous thrombolytic effect in the COW’s Y-shaped thrombus subregions (Figures 3 and 4; Figure V and Results in the online-only Data Supplement).

**Thrombolysis in the Cerebral Arteries Distal to the COW**

Prior to therapy, the thrombus burden in the distal arteries was 249×10³±34×10³ μm² for the tPA group and 148×10³±76×10³ μm² for the saline group (P=0.24). Though the distal cerebral arteries had significant thrombus reduction, the rate of reduction was generally lower, started later, terminated earlier, and showed a smaller initial thrombolytic effect than in the COW (Results in the online-only Data Supplement).

**Infarct Size and Relationship to Thrombus and Thrombolysis**

Ex vivo thrombus imaging and histological studies confirmed the tPA-mediated therapeutic effects on cerebral thromboemboli and infarcts, respectively. mCT imaging showed that saline-treated animals had ≈3.5-fold larger thrombus areas in the COW at 24 hours than the tPA-treated animals (488±94 versus 145±41 μm²; P<0.01). This was confirmed by ex vivo NIRF thrombus imaging (Figure 5A), which showed that tPA-treated animals had ≈3-fold larger COW thrombus areas than the control animals (505±91 versus 168±37 μm²; P<0.01). These results are biologically significant; as shown by 2,3,5-triphenyl-tetrazolium chloride staining, final infarct size at 24 hours was significantly larger in the saline group than in the tPA group (45.8±3.3 versus 18.1±2.3 mm²; P<0.01; Figures 1 and 5B). In addition, there was a strong correlation between thrombus areas as measured by mCT and NIRF imaging (r=0.81; P<0.01; Figure 5C, left). There was also a relatively strong correlation between infarct areas and mCT-measured COW thrombus areas (r=0.71; P<0.01; Figure 5C, middle). This correlation was stronger than that between infarct areas and NIRF imaging-measured COW thrombus areas (r=0.57; P<0.01; Figure 5C, right), likely because mCT was more accurate than NIRF imaging in estimating total thrombus burden. On the other hand, microscopic NIRF imaging could detect microvascular emboli, as well as bigger clots (Figure VI in the online-only Data Supplement).

**Infarct Size Was Smaller When Thrombus Clearance Occurred Earlier**

Within the tPA-treated cohort, final infarct size was smaller when complete thrombus clearance occurred earlier in the COW arteries (Figure 6). COW thrombi that were completely dissolved during hours 0.5 to 1 or hours 1 to 2 after the beginning of tPA infusion (n=7 and 5, respectively) had smaller infarcts (3.3±0.9 and 8.3±0.5 mm²) than those cleared between hours 1 to 2 or hours 2 to 3 (n=8, 13.3±1.2 mm²), respectively (all P<0.01; Figure 6). Further, infarct size was smaller when thrombus was cleared during hours 1 to 2 (n=5, 8.3±0.5 mm²) as compared with that during hours 3 to 24 (n=8, 17.1±2.0 mm²; P<0.01). However, thrombus clearance during hours 2 to 3 versus hours 3 to 24 hours did not show significantly different infarct sizes (P=0.13).

**Late Thrombus Clearance and tPA Use Alone Also Reduced Infarct Size**

When compared with tPA-treated animals without complete thrombus clearance in the COW by 24 hours (n=14, 32.2±4.3...
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mm²), tPA-mediated complete thrombus clearance beyond the therapeutic window (between 3 and 24 hours) did reduce infarct size significantly (n=8, 17.1±2.0 mm²; P<0.01). This result suggests that even late thrombus clearance can help reduce infarct size (Figure 6). Not one of the saline-treated animals (n=8) cleared thrombus by 24 hours, and their infarct sizes (45.8±3.3 mm²) were significantly larger, even when compared with the tPA group animals without thrombus clearance by 24 hours (P=0.04). It seems that even when thrombus failed to clear in tPA-treated animals within our measurement period, some residual benefit remains.

Among the animals without complete thrombus clearance in the COW by 24 hours, tPA-treated animals (n=14) had ≈30% smaller infarcts (32.2±4.3 mm²) than saline-treated animals (n=8, 45.8±3.3 mm²; P=0.04), although there were no significant intergroup differences in residual thrombus areas at 24 hours (0.43±0.08 versus 0.49±0.09 mm²; P=0.67). In addition, large infarcts that were bigger than the median value for the 22 animals (41.2 mm²) tended to be less frequently observed in the tPA group (5/14, 35.7%) than in the saline group (6/8, 75.0%; P=0.08). This, against expectations, suggests tPA may have a neuroprotective effect independent of thrombolysis.

Multivariable Analysis Showed That Infarct Size Was Smaller When Thrombus Cleared More Completely

mCT imaging detected not only residual thrombus in the COW but also scattered emboli in the distal vasculature across both hemispheres, particularly after tPA therapy (Figure 1, yellow arrowheads). Furthermore, multivariable analyses demonstrated that tPA (versus saline) treatment, thrombus clearance time, and residual thrombus burden in either the COW or the entire brain independently predicted the final infarct size (Table in the online-only Data Supplement).

Discussion

This is the first in vivo imaging study to directly visualize cerebral thromboemboli in a relatively large number of animals (n=50 mice) serially (at 8 time points/24 hours) in a quantitative manner by using high-resolution mCT and fibrin-targeted
AuNPs\textsuperscript{10} that we recently developed. No significant thrombus reduction could be observed until 1 hour after the initiation of tPA therapy at 1 hour after stroke. Most thrombolysis occurred within 3 hours of tPA therapy onset, with stronger observable effects within the 1- to 3-hour period. Thereafter, we noted further minor thrombus reduction until 24 hours, \( \approx 15\% \) (versus prior 85\%) of the total clearance, more so in the COW than in the distal arteries. By allowing quantitative assessments of early thrombolytic effect and residual thrombus burden, mCT imaging helped predict continued thrombolysis at later time points. Infarct size was smaller when thrombus was cleared earlier and more completely; however, late thrombus clearance and tPA use alone also reduced infarct size. Saline treatment was not associated with significantly decreased thrombus area.

Our novel understanding of the dynamic evolution of cerebral thromboemboli is highly significant, and if these findings translate to humans, our results would suggest time points for monitoring therapeutic success and provide thresholds—still within the therapeutic window—at which a lack of success would prompt additional treatment. In addition, our data indicate intersubject heterogeneity in the thrombus’s spatiotemporal response to tPA. This variable therapeutic response highlights the need for direct thrombus imaging to individualize thrombolytic therapy.

In our previous study, ex vivo NIRF imaging of brain tissue removed 24 hours after embolic stroke showed that thrombus burden in the COW closely correlated with final infarct volume as measured by 2,3,5-triphenyl-tetrazolium chloride staining.\textsuperscript{12} In the present study, using in vivo mCT-based direct thrombus imaging and fibrin-targeted AuNPs allowed us to (1) promptly detect cerebral thrombus, (2) accurately quantify thrombus burden, and (3) serially monitor thrombus evolution in response to fibrinolytic (versus saline) therapy. Further, multivariable analysis identified 3 independent predictors of final

![Image](image_url)

**Figure 4.** Thrombus-free rates in the subregions (Figure I in the online-only Data Supplement) of the circle of Willis at baseline and after the loading (designated as 0-hour time point) and 30-minute infusion of tissue-type plasminogen activator (tPA) in mice (n=42) with embolic stroke.

![Image](image_url)

**Figure 5.** Cross-correlations between microcomputed tomography (mCT)–based circle of Willis (COW) thrombus images, near-infrared fluorescent (NIRF) COW thrombus images, and infarct areas on 2,3,5-triphenyl-tetrazolium chloride (TTC) staining. A, In vivo mCT thrombus imaging and corresponding ex vivo NIRF thrombus imaging and TTC staining of the brain tissue. Infarct size was measured at 24 hours using 2-mm-thick TTC-stained sections at 0.98 (Section ii), −1.06 (Section ii), and −3.08 mm (Section iii) distance from the bregma. B, Infarct area. The final infarct size was significantly smaller in the tissue-type plasminogen activator (tPA)–treated animals than in the saline-treated animals. *\( P<0.05 \) and **\( P<0.01 \), Student’s \textit{t} test. C, Cross-correlations between the data sets (Pearson’s correlation). Scale bars =2 mm.
In the intravenous tPA-treated animals, but not the saline-treated controls, thrombolysis also occurred 3 to 24 hours posttreatment, which is far beyond the tPA’s 5- to 10-minute blood half-life. In addition to thrombolysis effectiveness and time point thereof, infarct size also varied because of residual thromboemboli burden at 24 hours, probably because of persistent distal arterial occlusion and compromised distal perfusion. Clinically, persistent distal emboli are frequently observed in mechanical thrombectomy cases. Moreover, small emboli that could be related to poor clinical outcomes may not be noticeable on traditional angiographic imaging. Distal flow disturbances can also be caused by stagnant flow in the distal microcirculatory bed; potential mechanisms for such stagnant
flow include activated platelet aggregation, leukocyte activation, local accumulation of inflammatory mediators, edema, and other factors that affect ischemic brain tissue, and endothelial function.\textsuperscript{6,17} These factors may explain why arterial recanalization does not always lead to brain tissue reperfusion.\textsuperscript{18} Recanalization and reperfusion are not synonyms and should not be used interchangeably in predicting infarct size.\textsuperscript{19}

Experimental studies on thrombolysis have demonstrated that effective delivery of fibrinolytic agents into thrombi by diffusion and convection is the most important determinant of fibrinolytic rate, which is highest when short clots are exposed to a high pressure gradient.\textsuperscript{20,21} In our study, the thrombolytic effects of tPA were relatively homogeneous for the 4 subregions of the Y-shaped COW thrombus. This observation suggests regionally consistent tPA delivery or subregional access to circulating plasminogen in the COW thrombus. If the proximal internal carotid artery had been severely narrowed or occluded (with collateral blood flow to anterior cerebral artery and middle cerebral artery being preserved or augmented), tPA-mediated thrombolysis may have been much slower and less complete in the distal intracranial internal carotid artery than in the other subregions of the COW. In addition to the hindered dissolution of clot, reduced perfusion pressure because of internal carotid artery steno-occlusion also causes hemodynamic instability, increasing the risk of rethrombosis after incomplete recanalization.\textsuperscript{22}

On the other hand, hemodynamic manipulations, such as triple-H (hemodilution, hypervolemia, and hypertension) therapy, might possibly allow improved access of tPA to thrombi, and imaging approaches likely allow the success of such therapies to be monitored. Again, CT and nanoparticle-based direct thrombus imaging could provide an early and objective readout of the success of such clinical manipulations.

In the arteries distal to the COW, a significant but lower thrombus reduction rate took longer to begin than in the COW itself. The relatively low thrombus reduction rate in the distal cerebral arteries may be partly because of thrombolysis-related distal embolism, which may have reduced the proximal thrombus burden while simultaneously increasing the distal thrombus burden.

Ribo et al\textsuperscript{15} showed that most tPA-induced recanalization occurred during the first hour after treatment, and patients with delayed flow improvement achieved 48-hour neurologically scores comparable to those of patients with early recanalization.\textsuperscript{8} Similarly, our animal study showed late thrombus clearance beyond the therapeutic window still significantly reduced infarct size. Against expectations, we found tPA to be therapeutically beneficial even in the absence of thrombus clearance, when compared with saline-treated animals, despite no significant intergroup differences in residual thrombus areas at 24 hours. This protective effect of tPA is both frequent, producing $\approx 50\%$ more relatively small infarcts, and significant, with infarcts $\approx 30\%$ smaller. These numbers dwarf the rates of symptomatic intracranial hemorrhage caused by tPA treatment in humans.

In the present study, embolic stroke was induced using erythrocyte- and fibrin-rich clots that were prepared as previously reported.\textsuperscript{23,24} Several studies showed that platelet-rich clots were relatively resistant to thrombolysis, highlighting the importance of clot composition on the efficacy of thrombolytic therapy.\textsuperscript{25} Platelet-rich clots may be better mimics of cerebral thrombi retrieved from a subset of stroke patients without recanalization after prior tPA therapy,\textsuperscript{25} suggesting a need for further investigation of in situ identification of clot structure to tailor treatment to the patient. Identification of such clots, possibly by using direct thrombus imaging to characterize thrombus composition, may allow early pursuit of endovascular therapy, such as mechanical clot retrieval.\textsuperscript{10,26}

In addition, fibrin-targeted glycol chitosan–coated AuNP CT imaging of large vessel occlusion–related thromboembolism is a powerful research tool for development of more rigorous and nuanced diagnostics and therapies for these clots. For example, platelet-rich clots may be more avidly sought out by platelet-targeted imaging agents than fibrin-targeted nanoparticles and might be better treated by therapeutics directed at platelets or fibrin as the case might be. Further studies are required to investigate if direct thrombus imaging using nanoparticles that target fibrin versus platelets could demonstrate differential efficacy of tPA versus eptifibatide or similar treatments.

Our experimental tPA study focused on thromboemboli causing macrovascular occlusion. For further research in the context of large vessel occlusion, it would be interesting to study the pharmacological effect of tPA or a novel drug that targets the thrombotic/inflammatory processes downstream of the culprit clot, the microvascular consequences of thromboembolism. Our study showed that mCT imaging after tPA therapy detected not only residual thrombi at the site of thromboembolic occlusion but also scattered emboli in the distal vasculature in vivo. Moreover, microscopic NIRF imaging of ex vivo brain sections could visualize microvascular emboli because of the fluorescent labeling of the culprit thrombi before being placed in the COW. Emerging experimental evidence suggests that tPA has substantial beneficial effects on the inhibition of downstream postcapillary microvascular thrombosis during occlusion and after recanalization.\textsuperscript{27}

Large vessel occlusion clots cannot be readily dissolved by tPA in the majority of cases, requiring endovascular therapy. However, the imaging approach we demonstrate in our study with tPA (feasible in rodents) could be used with equal effectiveness in the setting of endovascular therapy (feasible in man) and serves as a proof-of-concept for such future uses.

Despite the long-awaited success of endovascular therapy trials,\textsuperscript{28} there is much work remaining\textsuperscript{29,30} on (1) reducing delays to revascularization (because of poor vascular access or interhospital transfers), (2) increasing rates of complete revascularization,\textsuperscript{31} (3) decreasing the procedure-related complications (eg, distal embolization), (4) extending current indications with regards to the time window and ischemic territories, (5) customizing endovascular therapies (eg, selecting best devices for individual cases), and (6) determining the best methods for combined intra-arterial and medical approaches. All of these problems would benefit from improved imaging for triaging and monitoring the efficacy of current and future therapies (Discussion in the online-only Data Supplement).
In fact, imaging solutions might have a higher impact on the practice of endovascular therapy than on medical therapy in terms of the greater number of choices of devices and technical approaches needed. In addition, expanding the indications for endovascular therapy will likely lead to the inefficient use of angiography room resources without some prior triaging, to which direct thrombus imaging could contribute.

Intracranial atherosclerotic stenosis is one of the most common cause of stroke. In these patients with thrombosis caused by atherosclerotic stenosis, reperfusion may fail to be achieved by mechanical thrombectomy devices alone (Discussion in the online-only Data Supplement). Direct thrombus imaging that delineates thrombi from atherosclerotic vessel walls may enable an early procedural risk assessment to anticipate and avoid complications.

Despite limitations (Discussion in the online-only Data Supplement), this is the first study to describe and analyze, quantitatively and in detail, the spatial and temporal evolution of cerebral thromboemboli before and after the loading/infusion of tPA by means of a novel imaging technique. These data and AuNP direct thrombus imaging are likely to further the success of tPA and endovascular therapy by (1) enabling us to tailor thrombolytic therapy, (2) facilitating early or direct initiation of endovascular treatment, if implemented for prehospital triage in mobile stroke units with CT scanners, (3) supporting the better informed selection of specific reperfusion or endovascular therapies/devices, allowing for technical refinements, and better preparing rescue treatments, and possibly (4) guiding the recanalization therapy in near-real time with lowradiation flat-panel CT to track the intraprocedural evolution of thrombi during intra-arterial recanalization therapy.

Acknowledgments

We thank Jin-Yong Park for preparing and assisting in animal procedures.

Source of Funding

This work was supported by Global Research Lab (GRL) program (NRF2015K1A1A2028228) of the National Research Foundation, funded by the Korean government.

Disclosures

None.

References


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Stroke. 2017;48:1376-1385; originally published online April 21, 2017;
doi: 10.1161/STROKEAHA.117.016511
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/48/5/1376

Data Supplement (unedited) at:
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Supplemental Material

Quantitative Imaging of Cerebral Thromboemboli In Vivo
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Kim et al. High-resolution mCT Imaging of Thrombus Evolution

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Supplemental Introduction

Angiography identifies thrombus indirectly as a filling defect and cannot reliably assess thrombus load in a quantitative manner. In addition, angiographic evidence of arterial occlusion is lacking in 10–25% of patients with acute ischemic strokes,¹,² suggesting that some of these patients may have small distal emboli that are hard to detect. Moreover, angiography cannot distinguish between mural thrombus and atherosclerotic vessel wall, both of which could cause stenosis or occlusion.

Experimental use of optical direct thrombus imaging³⁻⁸ via intravital microscopy or intravascular catheters allows a sensitive visualization of thrombus at high-resolution in real time in vivo or ex vivo. However, because of poor depth penetration, these techniques do not allow a non-invasive in vivo assessment of thrombus load in the entire cerebral vasculature. Magnetic resonance imaging (MRI) or positron emission tomography (PET) based direct thrombus imaging has promise for clinical translation. However, these methods are expensive and have not shown good image contrast in rodents or humans.⁹⁻¹¹ Computed tomography (CT) remains the imaging modality of choice for the initial management of hyperacute ischemic stroke,¹² because of its wider availability and more rapid image acquisition when compared with MRI or PET.

To overcome many of the pitfalls that current thrombus-imaging techniques have, we have recently synthesized a fibrin-targeted glycol chitosan-coated gold nanoparticle (fib-GC-AuNP) and performed proof-of-principle experiments to characterize this CT-based imaging agent, focusing on its thrombus-visualizing capacity.¹³ In the present study, we quantified the evolution of cerebral thromboemboli using fib-GC-AuNP and microcomputed tomography, with/without tPA therapy.
Supplemental Methods

This study was approved by the Institutional Animal Care and Use Committee at Dongguk University Ilsan Hospital, and all experiments were conducted in accordance with the institutional guidelines on humane care and use of laboratory animals. Male C57BL/6 mice (n = 69, 10–12 weeks old) were purchased (Orient Bio, Seongnam, Korea) and maintained in a controlled environment at 23±0.2°C and 54±0.4% humidity, with 12 hours of light per 24-hour period. 19 mice were excluded because of subarachnoid hemorrhage while modeling stroke (n = 2), violation of experimental protocols (n = 8), or poor data quality (n = 9). Thus, 50 animals were included in this study. Based on our prior experiences, this sample size was considered proper for the microCT (mCT)-based imaging assessment of the dynamic evolution of thrombus after saline (n = 8) vs. tissue plasminogen activator (tPA, n = 42) treatment. Female mice were not used, because gender differences in post-tPA thrombus evolution were not prominent in our pilot experiments.

Synthesis of fib-GC-AuNPs
fib-GC-AuNPs were synthesized as previously reported. Briefly, GC dissolved in water (0.1 wt/wt%; 300 mL) was heated to 70°C, and then 100mL of HAuCl₄ (1 mmol/L) was added to the GC solution. The solution was maintained at 70°C with continuous stirring until the resulting GC-AuNPs were well suspended. Fibrin-targeting peptides (tyrosine-D-glutamine-cysteine-hydroxyproline-L-3-chlorotyrosine-glycine-leucine-cysteine-tyrosine-isoleucine-glutamine, 2.5 μmol/L) dissolved in dimethyl sulfoxide were activated with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride and N-hydroxysuccinimide, and this mixture was then added to the GC-AuNP solution and stirred for 4 hours at room temperature. Excess fibrin-targeting peptides were removed from the NPs by three rounds of centrifugation (9000 rpm, 45 minutes), washing, and resuspension.

Synthesis of the C15 near-infrared fluorescence (NIRF) thrombus marker
C15 NIRF imaging probes, which fluorescently label clots by binding to the fibrin matrix via the crosslinking activity of the FXIIIa coagulation enzyme, were synthesized as previously reported. Briefly, FXIIIa substrate peptides (glycine-asparagine-alanine-glutamate-glutamine-valine-serine-proline-leucine-threonine-leucine-leucine-lysine-tryptophan-cysteine) were synthesized using standard solid-phase Fmoc peptide chemistry (Peptron, Daejeon, Korea). Cy5.5 maleimide (2.5 μmol/L; excitation / emission = 675 nm / 695 nm; GE Healthcare, Little Chalfont, UK) were coupled with the thiol group on the cysteine of FXIIIa substrate peptides (2.5 μmol/L) in PBS (100 μL, pH 7.0) under stirring for 4 hours. The product was purified by reversed phase-high performance liquid chromatography (RP-HPLC) and lyophilized. Purity (> 95 %) was confirmed by RP-HPLC.

Mouse embolic stroke model
We used a mouse model developed to mimic embolic stroke in humans. In this model, we injected preformed fluorescently marked clots into the middle cerebral artery (MCA)–anterior cerebral artery (ACA) bifurcation area using a catheter placed in the distal common carotid
artery (CCA) following insertion through a hole made in the external carotid artery (ECA). The exogenously formed clot was labeled with the Cy5.5 fluorescent thrombus marker C15 probe.8 Mice were anesthetized using 2% isoflurane delivered through an inhalation mask. Cerebral blood flow was monitored using a laser Doppler flowmeter (LDF; Omegawave, Tokyo, Japan). Body temperature was maintained at 36.5°C using a homeothermic blanket (Panlab, Barcelona, Spain). A midline neck incision and dissection of the perivascular tissue were performed to expose the left CCA, ECA, and internal carotid artery (ICA). The ECA was ligated via coagulation at the origin of the superior thyroid artery. A microvascular clip (S&T AG, Neuhausen, Switzerland) was used to clamp the CCA, and the ICA was subsequently clamped after being separated from the adjacent vagus nerve. An arteriotomy was performed at the ECA proximal to the tying point. A tapered PE-50 catheter (0.15 mm in diameter; 15 mm in length) containing the clot was then inserted and maneuvered towards the ICA. The microvascular clip was immediately removed from the ICA, and the catheter was advanced approximately 9 mm toward the MCA-ACA bifurcation area and carefully removed once the thrombus was injected. Next, the ECA stump was ligated and the CCA was unclamped. For the inclusion of the animals in the final analysis, an a priori inclusion criterion should be met: LDF confirmed that cerebral blood flow was reduced to less than 30% of the baseline value.

In vivo mCT imaging and quantitative image analysis

Embolic stroke was induced as described above. One hour later, mCT images were acquired five minutes after intravenous administration of 300 μl fib-GC-AuNPs, as previously reported,13 using the following protocol: 65 kVp, 60 μA, 26.7×26.7×27.9 mm³ field of view, 0.053×0.053×0.054 mm³ voxel size, 500 milliseconds per frame, 360 views, 512×512 reconstruction matrix, and 600 slices. Based on a random allocation process run by a non-operating researcher drawing lots, these animals were then treated intravenously with either 1 mg/mL tPA (25 mg/kg, 600 μL) or saline (600 μL) bolus injection (10%), which was followed by continuous infusion (90%) for 30 minutes. Serial mCT images were acquired immediately after the bolus (designated as 0 hour), and at 0.5, 1, 1.5, 2, 3, and 24 hours after the bolus/start of the infusion.

mCT data were converted to the Digital Imaging and Communications in Medicine (DICOM) format to make three-dimensionally rendered images using a software package (Lucion, MeviSYS, Sound Beach, NY). Two study investigators (J.-Y.K. and S.-K.L.), who were strictly blinded to the treatment groups and imaging times, analyzed cerebral mCT images. Two sets of images were reconstructed for every imaging session in each animal: 1) one axial image corresponding to a 2 mm-thick basal brain region containing the entire circle of Willis (COW) thrombi and 2) six 2 mm-thick coronal images, corresponding to the 2,3,5-triphenyl-tetrazolium chloride (TTC)-stained brain sections at 2.96, 0.98, -1.06, -3.08, -4.60, -6.36 mm distance from the bregma, for assessing thromboemboli located above the level of COW at the base of the brain. Identically oriented and positioned follow-up images were prepared using nearby bony structures as anatomical references. In addition, to compare thrombus
burden and evolution in more detail, we divided the Y-shaped COW thrombus into four segments: ACA, MCA, posterior cerebral artery (PCA), and ICA (Supplemental Figure I).

Once the final versions of reconstructed cerebral or carotid image files were imported into Photoshop CS3-Extended (Adobe Systems, San Jose, CA), we performed background subtraction using a 40 pixels × 40 pixels square region drawn on an area of the brain parenchyma without any hyperdense thrombus-related lesions. The mCT images were then normalized using the ‘curves adjustment’ function in Photoshop in order to fix the background grayscale intensity values of the brain parenchyma and skull as 0 (black) and 255 (white), respectively, while preserving linearity. Thrombus-related lesions were segmented using the ‘wand’ tool, which automatically locates the edge of an object and traces its shape, allowing the area and mean AuNP-mCT density of the thrombus to be measured.

**Ex vivo NIRF imaging of cerebral thrombus and quantitative image analysis**

Once the in vivo mCT imaging was completed, the animals were euthanized, and the brains were removed and imaged using a NIRF imager with a charge-coupled device camera (CoolSnap-EZ, Roper-Scientific, Tucson, AZ). White light and Cy5.5 NIRF-channel (excitation / emission, 655 nm / 710 nm; 1-second acquisition) images were obtained on fresh whole-brain tissue or 2 mm-thick coronal sections. The ex vivo NIRF images underwent background subtraction and normalization, and thrombus-related Cy5.5 fluorescent areas were quantified as previously reported, using Photoshop CS3-Extended.

**Quantitative measurement of infarct size**

Three representative 2 mm-thick forebrain slices (at 0.98, -1.06, and -3.08 mm distance from the bregma) had the most extensive infarct involvement on TTC staining, and these slices were used consistently for infarct size quantification. As previously reported, whitish / pale infarct areas were segmented and final forebrain infarct size was measured.

**Statistics**

Inter-group differences were assessed using Student’s t-test. Friedman tests were used to compare related samples. Cochrane’s Q test was used to compare proportions. Pearson’s correlation coefficients were calculated to find a linear relationship between two continuous variables. Generalized estimating equation method and Bonferroni correction for multiple comparisons were used to compare repeatedly measured serial data: thrombus areas at two different time-points (‘adjacent’ or ‘baseline vs. later’ time-points) within each of the tPA and saline groups. Multiple linear regression analysis was performed to identify independent imaging predictors of cerebral infarct size. All statistical analyses were conducted using software packages (SPSS 18.0, IBM, Armonk, NY; SAS 9.4, SAS Institute, Cary, NC). A probability value of < 0.05 was considered statistically significant.
Supplemental Results

A) Saline treatment did not show significant change in the total thrombus area.

While about 54% of the pre-treatment baseline thrombus burden was cleared during the first 3 hours after tPA loading and infusion, saline treatment was not associated with significant thrombus change (corrected $P (P_{corr}) > 0.99$; Figure 1). During the 3–24 hour period, tPA treatment further decreased thrombus area by about 9.6% of baseline ($P_{corr} = 0.02$). Although a similar effect was also observed in the saline group ($6.8 \pm 5.3\%$ of its baseline value), this was not statistically significant ($P_{corr} = 0.67$ for 3 hours vs. 24 hours). In the saline group, thrombus areas at hours 3 and 24 were $687 \times 10^3 \pm 103 \times 10^3 \mu m^2$ and $625 \times 10^3 \pm 89 \times 10^3 \mu m^2$, respectively. Thus, unlike in the tPA group, thrombus areas in the saline group did not differ significantly before treatment and 24 hours later ($P_{corr} > 0.99$). In addition, there was a strong correlation between thrombus areas at baseline and 24 hours ($r = 0.83, P = 0.01$; Supplemental Figure II).

B) Thrombolysis in the COW and its subregions

Significant reduction of the COW thrombus area began 0.5–1 hours after the start of tPA therapy, with thrombolytic effect being strongest initially but continuing out to 24 hours.

The thrombus burden in the COW prior to therapy was $535 \times 10^3 \pm 43 \times 10^3 \mu m^2$ for the tPA group ($n = 42$) and $508 \times 10^3 \pm 105 \times 10^3 \mu m^2$ for the saline group ($n = 8, P = 0.80$). When thrombolytic therapy was initiated 1 hour after stroke, the thrombus area in the ipsilateral left cerebral arteries at the COW level did not significantly change until the 30-minute time point (Supplemental Figure IVA). Specifically, the thrombus area did not significantly reduce after tPA loading or during the subsequent 30-minute infusion (both $P_{corr} > 0.05$). At 1 hour after the start of tPA administration, the thrombus area became significantly smaller compared to pre-tPA baseline ($P_{corr} < 0.01$). This indicates a lag time between the start of tPA treatment and its effects: the first significant thrombolytic effect began in hour 0.5–1, with a thrombus reduction rate of $3810 \pm 826 \mu m^2/minute$ (Figures 1 and 2), as calculated by $[(\text{area}_{0.5h} - \text{area}_{1h})/\text{time}]$.

During the following 2 hours (Figure 2 and Supplemental Figure IVA), thrombus area reduction in the COW continued but at lower rates of change. More specifically, about 54–58% of the first significant thrombolytic effect occurred during hours 1–2 after tPA therapy began, and during hours 2–3 the rate of change dropped to 28%.

Thrombolysis continued at later time points, though much more weakly (Figure 2 and Supplemental Figure IVA). We observed continued thrombus area reduction out to 24 hours, but the rate of change was as low as $41 \pm 14 \mu m^2/minute$, which is only about 1% of the first, strongest thrombolytic effect observed in hours 0.5–1. During hours 3–24, however, a
considerable amount of COW thrombus was dissolved, with the thrombus area being significantly reduced from 196×10^3±44×10^3 μm^2 to 145×10^3±41×10^3 μm^2 (P corr = 0.01). In other words, of about 390 μm^2 thrombus (~73% of the baseline burden) dissolved in the 24 hours after the start of tPA therapy, about 87% was cleared during the first 3 hours, whereas the other 13% was cleared during the next 21 hours.

**tPA showed a largely homogeneous thrombolytic effect in the COW’s Y-shaped thrombus subregions.**

In the tPA group, mean thrombus areas (×10^3 μm^2) were similar across all four segments of the COW (P = 0.70, Figure 3): ACA (1212±173), PCA (1245±148), MCA (1188±115), and ICA (1314±128). In each segment, thrombus area was significantly reduced in hours 0.5–1, and the reductions during this period tended to be relatively high compared with all later periods (Figure 3 and Supplemental Figure V). The initial and maximal thrombus reduction rates were not significantly different across the segments (P = 0.79): 1222±420 (ACA), 976±339 (PCA), 817±240 (ICA), and 675±252 (MCA) μm^2/minute.

Thrombus-free rates at baseline were significantly different (varying from about 2 to 20%) across the four COW subregions (P = 0.04), with relatively high frequencies in the ACA (19.0%, 8 of the 42 tPA-treated animals) and PCA (14.3%, 6/42) compared with the MCA (4.8%, 2/42) and ICA (2.4%, 1/42; Figure 4). Thereafter, the subregional frequencies gradually increased: about 30–40% by 1 hour, 60–70% by 3 hours, and finally 70–80% by 24 hours. Thrombus-free rates at 24 hours were similar across the subregions (P > 0.05), with ACA at 81.0% (62.0% absolute increase from the baseline value), PCA at 73.8% (59.5% increase), MCA at 73.8% (69.0% increase), and ICA at 71.4% (69.0% increase). Thus, the absolute increases of the subregional thrombus-free rates (by 24 hours from baseline) were about 10% higher in the MCA and ICA than in the ACA and PCA.

**The final COW thrombus burden 24 hours after the start of tPA therapy could be predicted by mCT-measured thrombolytic effect at 1.5–2 hours and residual thrombus burden at 2 hours.**

There was no significant correlation between the initial significant thrombolytic effect and any of the subsequent thrombus reduction rates at hours 1–1.5, 1.5–2, 2–3, or 3–24 hours (all P > 0.05; data not shown), thereby indicating that the earliest evidence of thrombolysis in the COW did not predict continued thrombolysis at later time points. However, there was a modest correlation between the thrombus reduction rates at hours 1.5–2 and 3–24 (r = 0.54, P < 0.001; Supplemental Figure VIIA), and this correlation shows that relatively early evidence of thrombolysis may predict continued thrombolysis in the COW. Moreover, the linear associations between the thrombus area at 24 hours and at previous time points (i.e. baseline–3 hours) tended to strengthen over time but plateau at 2 hours (Supplemental Figure VIIIB). COW thrombus burden measured by mCT images 2 hours after the start of tPA therapy could
strongly predict thrombus burden at 3 hours \( (r = 0.91, P < 0.01; \text{Supplemental Figure VIIC}) \) and 24 hours \( (r = 0.87, P < 0.01; \text{Supplemental Figure VIID}) \).

**Saline treatment significantly increased the COW thrombus area during the first 3 hours.**

During the first 3 hours after tPA loading and infusion start, 68.2±6.3% of the baseline thrombus area was cleared (Figure 1). However, saline treatment was associated with significantly increased COW thrombus area \( (8.5±2.7\% \text{ of baseline}; P_{corr} < 0.01) \). In hours 3–24, tPA treatment further decreased 7.9±2.4\% of the pre-tPA baseline thrombus area. A similarly delayed thrombolysis also occurred in the saline group \( (7.7±5.4\% \text{ of baseline}) \), but this was not statistically significant \( (P_{corr} > 0.99 \text{ for 3 hours vs. 24 hours}) \). Thus, unlike in the tPA group, thrombus area in the saline group did not differ significantly between the pre-treatment baseline and the 24-hour time point \( (P_{corr} > 0.99) \). In addition, there was a strong correlation between thrombus area at baseline vs. 24 hours in the saline group \( (r = 0.88, P = 0.004; \text{Supplemental Figure VIII}) \).

**C) Thrombolysis in the cerebral arteries distal to the COW**

Though the distal cerebral arteries had significant thrombus reduction, the rate of reduction was generally lower, started later, terminated earlier, and showed a smaller initial thrombolytic effect than in the COW.

When thrombolytic therapy was initiated 1 hour after stroke, the mean area of thrombus in the cerebral arteries distal to the COW did not change significantly until the post-tPA 1-hour time-point (Figure 1). 1.5 hours after the start of tPA treatment, the thrombus area became significantly smaller than the pre-tPA baseline. This indicates that tPA therapy effects occurred relatively later in the distal arteries than in the COW (at 1.5 hours vs. 0.5–1 hour). Moreover, the rate of thrombus reduction in the distal arteries was 794±276 \( \mu \text{m}^2/\text{minute} \) (Figure 2), which is about 20\% of the rate seen in the COW (3810±826 \( \mu \text{m}^2/\text{minute} \)). Pre-tPA thrombus burden in the distal arteries was about a half of that in the COW (Supplemental Figure IV). Thus, the disproportionately low post-tPA thrombus reduction rate still suggests a relatively low net thrombolytic effect in the distal arteries.

Thrombus area reduction in the distal arteries continued at relatively low rates but without statistical significance for each of the four pre-determined time periods (Figure 2). Compared with the initial rate, the thrombus reduction rate in the 1–1.5 hour period was about 13\% lower (i.e. 87\% level) in the distal arteries than in the COW, followed by about 79, 30, and 2\% levels at 1.5–2, 2–3, and 3–24 hours, respectively. During hours 3–24, the thrombus area decreased from \( 163\times10^3±25\times10^3 \mu \text{m}^2 \) to \( 142\times10^3±25\times10^3 \mu \text{m}^2 \), which was, however, not statistically significant \( (P_{corr} = 0.36; \text{Supplemental Figure IVB}) \).
The final thrombus burden in the distal arteries 24 hours after the start of tPA therapy could be predicted by mCT-measured thrombus burden at 2 hours. In the cerebral arteries distal to the COW, there were no significant correlations between the initial thrombolytic effect at 0.5–1 hour and the subsequent thrombus reduction rates (data not shown). Yet there were weakly or modestly ‘positive or negative’ (i.e. directionally inconsistent) correlations between the thrombolytic effect at 1–1.5 hours and all subsequent thrombus reduction rates at hours 1.5–2 ($r = -0.56, P < 0.01$), 2–3 ($r = 0.36, P = 0.02$), and 3–24 ($r = -0.56, P < 0.01$) (Supplemental Figure IX).

The linear associations between the thrombus areas at 24 hours and prior time-points (i.e. baseline–3 hours) tended to gradually strengthen over time and plateaued at around 2 hours (Supplemental Figure IXD). Thus, mCT-measured thrombus burden 2 hours after the start of tPA therapy strongly predicted thrombus burden at 3 hours ($r = 0.87, P < 0.01$; Supplemental Figure IXE) and at 24 hours ($r = 0.82, P < 0.01$; Supplemental Figure IXF).

Saline treatment was not associated with significant change in the thrombus area of the distal arteries. While about 35% of the baseline thrombus burden in the cerebral arteries distal to the COW cleared during the first 3 hours after tPA loading and infusion, saline treatment did not significantly change the thrombus area ($P_{corr} > 0.99$; Figure 1). Later, i.e. during hours 3–24, neither tPA ($P_{corr} = 0.36$ as mentioned above) nor saline treatment ($P > 0.99$) significantly changed the thrombus area. In the saline group, thrombus areas at hours 3 and 24 were $145 \times 10^3 \pm 68 \times 10^3 \mu m^2$ and $138 \times 10^3 \pm 68 \times 10^3 \mu m^2$, respectively. Unlike in the tPA group, thrombus areas in the saline group did not differ significantly between the pre-treatment baseline and the 24-hour time-point ($P_{corr} > 0.99$). In addition, there was a very strong correlation between thrombus areas at baseline vs. 24 hours ($r = 0.99, P < 0.01$; Supplemental Figure X).
Supplemental Discussion

All current evidence convincingly shows that the recanalization efficiency is too low for tPA alone in cases of high thrombus burden / large-vessel-occlusion (LVO), but that good recanalization rates are achieved with combined intravenous tPA and endovascular treatment, without additional risk from adding the endovascular component. Although our experimental study focused on thromboemboli causing ‘macrovascular’ occlusion and treating these with tPA, the future prospect of direct thrombus imaging should not be underestimated for use in the endovascular setting. The novel imaging technique we describe is agnostic to treatment modality, and allows for monitoring cerebral thromboembolism and its evolution after any type of therapy. We believe that direct thrombus imaging will help to advance the field of vascular neurology by supplying better quality information to guide research and therapeutic decisions.

Direct thrombus imaging can characterize thrombus burden and composition in a prompt manner, potentially allowing endovascular treatment resources to be used in a more rational manner. When translated into clinic, our novel imaging method may guide thrombolytic therapy by enabling clinicians to reduce tPA dose for smaller fragile thrombi and perform endovascular clot retrieval more aggressively (e.g. proceeding directly to endovascular therapy) for bigger compact thrombi that are likely to be highly resistant to conventional tPA doses.

Poor vascular access or interhospital transfer can cause delay or failure in endovascular therapy. Endovascular therapy as a stand-alone treatment without intravenous thrombolysis may suffer from missing potential tPA respondents even among LVO patients: such as the ones with acute LVO due to a tiny in situ thrombus or embolus superimposed on a cerebral artery with significant large artery intracranial atherosclerotic stenosis (ICAS). A recent study on LVO thrombosis due to ICAS showed that retrieved thrombi were generally quite small because of the relative contribution of atheroma, rather than clot to the occlusion. It is notable that direct thrombus imaging could differentiate thrombus from atheroma with high sensitivity, unlike most of current methods such as MR or CT angiography visualizing thrombus indirectly by confirming the obstruction of arterial blood flow.

Even those LVO patients who receive endovascular therapy directly from the beginning in the hospital spend time for transportation, allowing room for prehospital usage of thrombolytics. tPA treatment in the ‘3- to 6-hour’ window had worse outcomes compared to placebo-treated patients with ICA occlusion in the Echoplanar Imaging Thrombolytic Evaluation Trial (EPITHET). However, in a case-control multicenter study performed in 27 centers in 7 countries, intravenous tPA treatment within ‘4.5 hours’ from stroke onset significantly increased the proportion of independent patients with 3-month modified Rankin Scale score of 0-2 in 253 consecutive patients with acute ischemic stroke and ICA occlusion (28.9%) vs. 20.6% of 253 (age/gender/stroke-severity) matched controls with acute ischemic stroke and ICA occlusion not treated with tPA (adjusted odds ratio, 1.80, \( P = 0.037 \)).
Although increases in death and intracranial hemorrhage, possibly due in part to reperfusion-associated damage, were the trade-offs for this clinical benefit, early tPA treatment should not be abandoned in LVO patients until proven to be unnecessary in a prospective randomized study. In addition, earlier or prehospital (vs. inhospital conventional time-window) treatment with tPA or new thrombolytics might show relatively high re-canalization efficiency in LVO stroke (or even in cases with high thrombus burden), to be confirmed in future investigations.

There are still theoretic concerns over the safety of endovascular devices. Mokin et al. argued that aspiration thrombectomy could be highly effective in embolic LVO, whereas using stent retrievers might be of more benefit intracranial or extracranial atherosclerotic stenosis-related thromboembolism. In fact, ICAS is the most common cause of stroke worldwide. It has been reported that recanalization failure and mortality after thrombectomy using stent retrievers are high in patients having atherothrombotic lesions. Intracranial atheromata can cause arterial narrowing and tortuosity, and constrain effective delivery of thrombectomy devices. Moreover, they can resist sufficient expansion and retraction of stent retrievers at the steno-occlusive lesion, requiring forceful maneuvers, which may in turn increase the risk of acute endothelial damage or plaque rupture leading to thrombotic reclosure as well as the risk of arterial rupture, dissection, vasospasm, or tearing of perforator vessels.

Development of an early assessment method, such as direct thrombus imaging, to better characterize the type of occlusion, will allow physicians to: a) select their tools and techniques with better information, b) anticipate potential complications and c) the likelihood of the need for rescue / secondary treatments to be needed. Direct thrombus imaging has the benefit of delineating thrombi as separate from the atherosclerotic vessel walls, and thus may help physicians to distinguish cases with an underlying atherosclerotic lesion from those that are purely embolic in nature. This critical distinction will enable clinicians to better select specific endovascular therapies / devices and allow for technical refinements, leading to safer and more effective endovascular therapy.

It has been suggested that future research should be also directed at increasing the roles of emergency medical services and how best to expand the indications of endovascular therapy for acute stroke. An increased number of patients receiving the intervention will likely lead to inefficient use of angiography room resources without more advanced methods for better prehospital or emergency department triage. It is not yet clear which imaging modality will prove to be of the highest value for performing this triage.

A recent meta-analysis showed that Thrombolysis In Cerebral Ischemia (TICI) III recanalization rate was about 52% by endovascular therapy (vs. about 26% by tPA). The well-known impact of residual thrombus burden on re-occlusion of re-canalized arteries warrants further investigation of residual thrombus elements at thromboembolic LVO sites. In a similar vein, there is an argument that future trials should adopt more stringent efficacy
endpoints than TICI IIb/IIIa. Direct thrombus imaging enables assessment of the completeness of thrombolysis at thromboembolic sites, and detects prognostically important residual thrombus elements that might be missed with more traditional imaging techniques.

After the success of stent retrievers in mechanical embolectomy, several new devices are being developed to offer improved recanalization and minimize distal clot embolization.\textsuperscript{32} For example, it was suggested that aspiration thrombectomy with recently developed, new generation distal aspiration catheters might improve recanalization rates.\textsuperscript{19} As was the case in the field of interventional cardiology, clinical trials with head to head comparisons of endovascular devices and techniques will be required to identify optimal approaches. Such trials will greatly benefit from the accurate definition of biologically similar treatment arms by means of novel imaging techniques such as the ones we describe.

Thromboembolic stroke models provide less control over the location and extent of the resulting cerebral infarction than other, less physiologic models.\textsuperscript{8, 33, 34} The resulting variability in stroke, ranging from little damage to death is well known, and is reflective of the clinical realities of human practice. We have previously demonstrated that the thromboembolic stroke model used in this study, although a faithful mimic of infarcts in human patients, is beset by the same heterogeneity of tissue outcome observed in humans.\textsuperscript{8} However, we also showed that direct optical thrombus imaging, combined with LDF, could a) characterize the highly heterogeneous nature of the stroke model, and b) control for infarct variability by stratifying animals to select for a subset of homogenous infarcts that were suitable for the detection of subtle therapeutic effects.\textsuperscript{8}

Unlike the autologous clot model that we used, a different model of thromboembolic stroke could have been induced by in situ microinjection of thrombin into a branch of the MCA with good precision,\textsuperscript{35} providing more reproducible size and location of cortical infarction with minimal mortality.\textsuperscript{33} However, this is counterbalanced\textsuperscript{33, 34} by: a) the need for a craniotomy and dura excision to expose the MCA branch, b) the need for arteriotomy to insert the tip of a thrombin-filled micropipette into the blood vessel, both these interventions add new variables, and in addition, c) this model lacks a robust neurological deficit, because of the small cortical lesions it produces. Lastly, d) it has been suggested that the supra-physiological thrombin content in clots produced by this model may confer resistance to currently available thrombolytic agents beyond what is evident in the clinical practice,\textsuperscript{36} thus making this model less suited for evaluating tPA based interventions.

Our study builds on / differs from other researchers’ work in several significant ways. For example, using transcranial Doppler, Alexandrov et al\textsuperscript{37} reported that post-tPA recanalization began as early as about 20 minutes and concluded about 40 minutes after treatment initiation in a hospital-based cohort (n = 65 patients) with heterogeneous arterial occlusion sites (M2 MCA segment 21%, M1 MCA 49%, tandem MCA and ICA occlusion 16%, terminal ICA 5%, and vertebrobasilar vessels 9%).\textsuperscript{37} The present study, by contrast, investigated a relatively high tPA dose (25mg/kg, which is about twice the usual dose for rodents) on ICA-T
occlusions (ICA plus MCA and ACA) in mice. Furthermore, we directly and serially imaged thrombus to assess volume reduction rather than continuously monitoring flow to confirm recanalization.

During the present study, tPA treatment began as early as one hour after stroke onset, and we did not observe hemorrhagic transformation. Thus, our tPA-related hemorrhagic experience does not reflect clinical realities in humans, as symptomatic intracranial hemorrhage occurs in about 6% of human patients undergoing tPA treatment. This gap between our animal model and clinical outcomes suggests that further studies are required to investigate the potential neuroprotective effects of tPA, as well as its well-described neurotoxic effects, with stratification for two important confounding variables: a) tPA-mediated thrombus volume reduction that affects perfusion / reperfusion status and final ischemic damage of the brain and b) post-tPA blood-brain barrier (BBB) dysfunction that promotes hemorrhagic transformation complicating ischemic infarct. This type of complex, demanding investigation is challenging but will likely be feasible using mCT-based serial in vivo imaging that combines the current direct thrombus imaging technique with a high-resolution BBB imaging technique that we recently developed.

Despite the limitations, this is the first study to quantitatively assess a detailed spatial and temporal evolution of cerebral thromboemboli after tPA therapy. We also demonstrated that high-resolution mCT-based direct thrombus imaging using thrombus-seeking nanoparticles could provide an early and objective readout of thrombolytic success. This novel thrombus imaging technique could visualize cerebrovascular thromboemboli precisely and monitor thrombolytic therapy in near-real time, and will likely contribute to the future development of new thrombolytics, or new treatment regimens with existing therapeutic agents, due to the following advantageous characteristics: a) high spatial resolution (~50 μm), b) good temporal resolution (several minutes), c) no background noise, and d) affordable costs. The implications for clinical translation of this new method and for the provision of feedback allowing adaptive, personalized treatment of stroke, are obvious.
Supplemental Figure I. Circle of Willis (COW) thrombus and its four segments on mCT images: anterior cerebral artery (ACA), middle cerebral artery (MCA), posterior cerebral artery (PCA), and internal carotid artery (ICA).
Supplemental Figure II. A strong correlation between thrombus areas at baseline and 24 hours in the saline group.
Supplemental Figure III. Relationships between early and late time-points in terms of thrombus reduction rates (A) and thrombus areas (B-D) in the entire brain after the start (designated as 0 hour time-point) of intravenous administration of tissue plasminogen activator (tPA).
Supplemental Figure IV. Quantification data of the horizontal view mCT images at the circle of Willis (COW) level (A), and those of the coronal view images (with the thrombus in the circle of Willis excluded in the calculation of thrombus area, B). Tissue plasminogen activator (tPA) reduced the amount of thrombus in the COW over time (A) and reached significance 0.5 hours after the end of the 30 minute infusion (i.e. 1 hour after tPA treatment initiation and 2 hours after embolic stroke vs. baseline). Thereafter, thrombus area reduced further out to 24 hours (vs. 3 hour time-point). There was no significant change in the thrombus area during the same period in the saline-treated animals. Moreover, saline treatment was associated with significantly increased thrombus area (2 vs. 3 hour time-point). Quantification of coronal view image data (B) shows that the thrombus amount in the area distal to the COW began to be significantly reduced by 1.5 hours (vs. baseline) in the tPA group but not in the saline group. There was no significant thrombus area reduction between 3 and 24 hours in either group. Graphs show mean ± SEM. *P < 0.01, †P < 0.05, and &P < 0.1 by generalized estimating equation method and Bonferroni correction for multiple comparisons between thrombus areas at two different time-points (‘adjacent’ or ‘baseline vs. later’ time-points) within each of the tPA and saline groups. Red and blue lines connect the means. Scale Bars = 2 mm
Supplemental Figure V. Thrombus area changes (before – after; $\times 10^3 \mu m^2$) measured over 24 hours in the circle of Willis subregions in mice (n = 42) with embolic stroke treated by intravenous loading (designated as 0 hour time-point) and 30-minute infusion of tissue plasminogen activator. Graphs show mean ± SEM. *$P < 0.01$, #$P < 0.05$, and &$P < 0.1$ by generalized estimating equation method and Bonferroni correction for multiple comparisons between the repeatedly measured serial data. Pink lines connect the mean values after adjustment for the longer time intervals of 2–3 hours and 3–24 hours.
Supplemental Figure VI. Ex vivo microscopic near-infrared fluorescent imaging to visualize microvascular emboli (yellow arrows, inlet for a magnified view; upper image) dislodged from the culprit thrombus (white arrow, upper image) that were pre-labeled with coagulation factor XIIIa-sensing Cy5.5 probe before being placed in the circle of Willis. When non-labeled thrombus was used for generating stroke, neither thrombus nor microvascular emboli are visible (lower image).
Supplemental Figure VI. Relationships between early and late time points in terms of thrombus reduction rates (A-B) and thrombus areas (C-D) in the circle of Willis (COW) after the start (designated as 0 hour time-point) of intravenous administration of tissue plasminogen activator.
Supplemental Figure VIII. A strong correlation between thrombus areas at baseline and 24 hours in the circle of Willis of the saline group.
Supplemental Figure IX. Relationships between early and late time-points in terms of thrombus reduction rates (A-C) and thrombus areas (D-F) in the arteries distal to the circle of Willis after the start (designated as 0 hour time-point) of intravenous administration of tissue plasminogen activator (tPA).
Supplemental Figure X. A strong correlation between thrombus areas at baseline and 24 hours in the arteries distal to the circle of Willis of the saline group.
Supplemental Table. Multivariable analyses to predict triphenyl-tetrazolium chloride (TTC)-measured infarct size (mm\(^2\)) at 24 hours using mCT thrombus imaging data.

<table>
<thead>
<tr>
<th>Variable</th>
<th>B (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Model I</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tPA treatment (vs. saline treatment)</td>
<td>16.1 (7.9 – 24.3)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>1 / COW-thrombus clearance time (per 1/hour)</td>
<td>-19.0 (-27.9 – -10.1)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Total residual thrombus burden at 24 hours (per mm(^2))</td>
<td>16.9 (7.8 – 26.1)</td>
<td>0.001</td>
</tr>
<tr>
<td><strong>Model II</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tPA treatment (vs. saline treatment)</td>
<td>14.4 (6.1 – 22.7)</td>
<td>0.001</td>
</tr>
<tr>
<td>1 / COW-thrombus clearance time (per 1/hour)</td>
<td>-18.2 (-27.1 – -9.2)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Residual thrombus burden in the COW at 24 hours (per mm(^2))</td>
<td>22.5 (10.9 – 34.1)</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

tPA and COW denote tissue plasminogen activator and circle of Willis, respectively.
P < 0.001 / r\(^2\) = 0.70 and P < 0.001 / r\(^2\) = 0.71 for models I and II, respectively.
When thrombus is not cleared by 24 hours, ‘1 / Thrombus clearance time’ is 0.
References


