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Histopathological Characteristics of IV Recombinant Tissue Plasminogen -Resistant Thrombi in Patients with Acute Ischemic Stroke

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Introduction

Rates of angiographic recanalization following IV recombinant tissue plasminogen activator (rt-PA) vary from 23% to 56% depending upon the location of occlusion and time interval between administration of rt-PA and ascertainment of recanalization [1–3]. It remains unclear whether certain thrombus characteristics result in resistance to lysis with IV rt-PA. In animal models of embolic stroke and femoral artery thrombosis, white thrombi composed of platelets and fibrin displayed a relative resistance against thrombolysis, whereas erythrocytes-rich (red) thrombi were more vulnerable to lysis [4]. Platelet-rich thrombi were relatively resistant to rt-PA compared with in a rabbit model of femoral artery thrombosis [5]. Furthermore, thrombolytics exposure results in platelet activation, which increases the platelet content in thrombus and thus render thrombus resistant to additional thrombolytic activity [6].

We performed this study to evaluate and compare the histopathological characteristics of persistent *in vivo* thrombi retrieved from acute ischemic stroke patients who had received IV rt-PA prior to endovascular treatment.

Methods

A retrospective study of consecutive patients with acute ischemic stroke who underwent endovascular treatment was performed. The institution maintained a prospective endovascular procedure database, which records information regarding the procedural components. All procedural records including devices used and intraprocedural medication with doses are archived into an electronic medical record system. The protocol for collecting data was reviewed and approved by the Institutional Review Board at the institution as part of a standardized database.

Data Collected

We recorded the presence of cardiovascular risk factors (active cigarette smoking, hypertension, atrial fibrillation, coronary artery disease, hyperlipidemia, diabetes mellitus, history of aspirin use, prior transient ischemic attack [TIA] or ischemic stroke), time interval between symptom onset and endovascular treatment, and use of IV rt-PA. IV rt-PA was administered using the protocol recommended by American Heart Association/American Stroke Association [7]. The times were identified individually by reviewing the angiographic images and recording the times of IV rt-PA initiation and deployment of retrieval system. We also recorded the time interval between symptom onset and deployment of retrieval system. We also recorded admission, 24-hour post-treatment, and discharge National Institutes of Health Stroke Scale (NIHSS) scores. Angiographic occlusion and recanalization were classified by interventional neurologists by using the previously validated grading scale [8,9]. Complete recanalization was defined as a grade of 0 and partial recanalization was defined by a reduction in severity of occlusion by 1 grade or greater.

Outcomes at the time of discharge were assessed by using the modified Rankin scale (mRS), ascertained from hospital discharge summaries and outpatient visit records by the vascular neurology team and occupational, speech, and physical therapists. The principal safety end points were intracerebral hemorrhage (ICH) and in-hospital mortality. Symptomatic ICH was defined as noncontrast CT–documented ICH resulting in neurologic deterioration (≥4-point worsening on an NIHSS score compared with previous clinical assessment). Favorable functional outcome at discharge was defined by a mRS score of 0–2.

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Protocol for thrombus retrieval

6 Fr Envoy guiding catheter (Cordis, Miami Lake, FL USA) was introduced through a femoral sheath into the appropriate carotid artery or dominant, or navigable, vertebral artery over a 0.035-inch steerable guidewire using fluoroscopic guidance supplemented by road mapping technique. Infrequently, an 8 Fr or 9 Fr Merci balloon guiding catheter (Concentric Medical, Mountain View, CA, USA) was used. In a typical procedure, Prowler PLUS 0.021-inch two-tip microcatheter was advanced over the steerable 0.014-inch guidewire (SYNCHRO-II guidewire). Radiopaque marker bands located at distal tip of the catheter were used to position the microcatheter under fluoroscopic visualization across the site of occlusion. A microcatheter angiographic injection was then carried out in order to confirm and define the vasculature distal to the thrombus. The Solitaire FR revascularization device (EV3, Irvine, CA, USA) 4 × 20 mm was then introduced through the microcatheter via a 0.016-inch nitinol push wire deployed across the occluding thrombus. The Solitaire FR device was maintained in place for 7–10 min to allow device expansion and integration within the thrombus. Subsequently, the fully deployed Solitaire FR and the delivery microcatheter were retracted together and recovered through the guiding catheter. Infrequently, a 2.5 F MC18 Plus microcatheter (Concentric Medical Inc) was navigated over a 0.014 microwire across the occlusive thrombus. The Trevo device was then advanced into the microcatheter spanning the whole length of the occlusive thrombus. A similar procedure as described for Solitaire FR revascularization device was performed. Infrequently, Alteplase (1 mg/ml) was infused through manual injection using 3-cc syringe through the microcatheter in bolus doses of 2–3 mg at several intervals prior to and after stent retriever device deployment and retrieval. The status of recanalization was assessed at regular intervals through angiographic images acquired in both antereoposterior and lateral projections. Intravenous heparin was not administered in any patient. A continuous flush system of 0.9% sodium chloride with heparin (1000 U in 1 L) was used to irrigate the guide catheter, femoral introducer sheath, and microcatheter (when required). During the retrieval, manual aspiration with a 50-mL syringe was performed through the hemostatic valve during the retrieval in order to reverse the flow and to aspirate thrombus debris present in the lumen of the guide catheter.

Processing of Extracted Thrombi

The thrombus was removed from the device or retrieval catheter by gentle disassociation without any dilution with any solvent. Subsequently, the thrombus was placed in a specimen cup with 10% neutral buffered formalin for fixation. The tissues selected were processed for paraffin section preparation. Specimens were carefully orientated to allow visualization of center of thrombus. Paraffin sections were cut at a thickness of 3–5 µm ensuring that only a single layer of cells makes up the section. The sections were deparaffinized and rehydrated. The sections were stained by hematoxylin and eosin (H/E) staining method which involved immersion into Harris hematoxylin solution for 8 minutes followed by counterstain in eosin–phloxine solution for 1 minute. The sections were mounted with xylene-based mounting medium. The thrombi sections were processed and slides were washed and counterstained with H/E stain (200x), observed under light microscopy. The erythrocytes were identifiable by discoid shape without any nuclei and fibrin was identifiable was pink amorphous deposits within the thrombus. The thrombi were further classified as erythrocytes aggregates, or erythrocytes nests with fibrin mesh in repeating patterns or in random patterns. The pattern was defined by alternating light and dark lines (lines of Zahn) which represent bands of fibrin (lighter) with entrapped white blood cells and platelets and erythrocytes (darker) [10].

Quantitative Thrombus Composition Analysis

We identified the following four distinct regions within the thrombus: dense erythrocytes aggregates; mixed region consisting of dense eosinophilic amorphous material deposits with intermingled erythrocytes; eosinophilic amorphous material deposits; and basophilic staining cells in linear aggregates intermingled within the three above-mentioned regions. Thrombus type was defined as erythrocytes, fibrin, or mixed region dominant based on the component that occupied the largest proportion of thrombus. Hematoxylin and eosin-stained slides were scanned at 400x magnification using a LAS EZ (Leica microsystem, Bufflogrove, IL) digital scanner. Image J software (National Institutes of Health, Bethesda, MD) was used to quantify the four distinct areas as mentioned above based on automated thresholds to assign specific colors to imaging features of each thrombus component (Figs. 1–4). The area of each region was expressed as percentage of total thrombus area in slide. In cases where multiple thrombus fragments were retrieved for analysis, the mean values across fragments were used for thrombus regions.

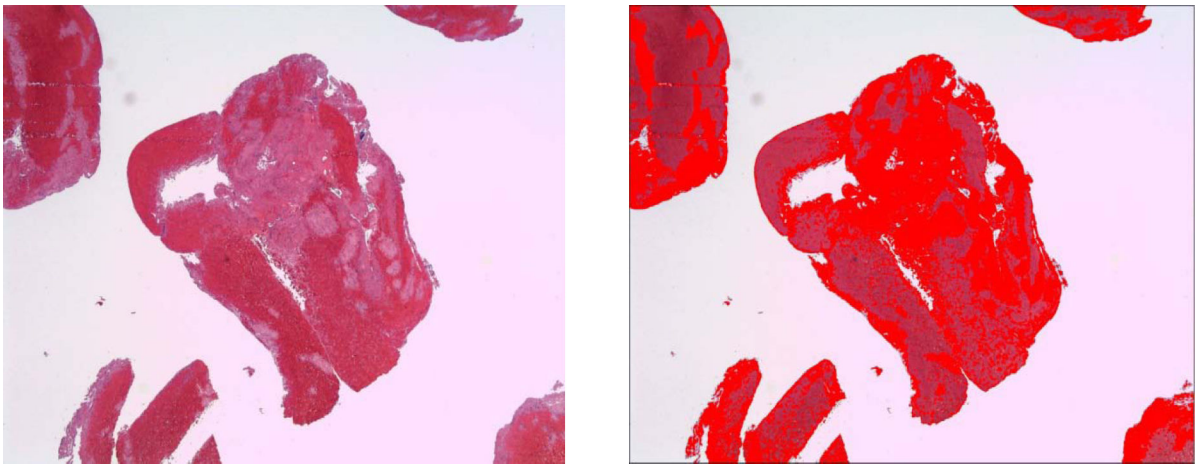

Figure 1. The mixed region in a rt-PA naïve thrombus (A) which is highlighted by automated thresholds to assign specific colors to clot component (B)

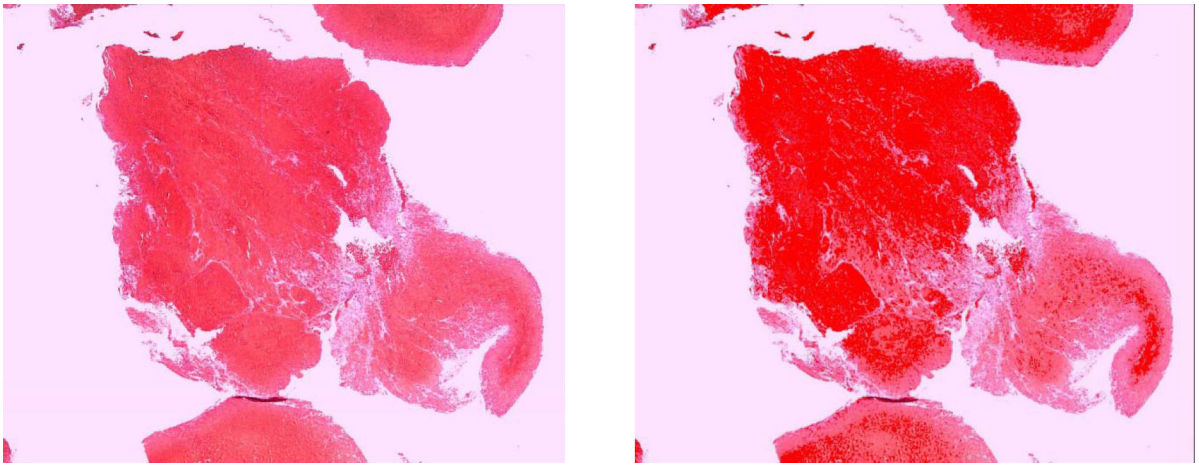

Figure 2. The erythrocyte aggregates in a rt-PA-resistant thrombus (A) which is highlighted by automated thresholds to assign specific colors to clot component (B)

Statistical Analysis

The analysis was predominantly descriptive with categorical and continuous variables expressed as frequencies and mean with standard deviations, respectively.

Results

A total of 24 patients (mean age ± SD of 73.1 ± 14.4 years) underwent mechanical thrombectomy during the study period, out of them thrombi was retrieved from 18 patients. IV rt-PA was administered prior to endovascular treatment in seven of 18 patients. Thrombi were retrieved from the 18 patients using either solitaire (n = 16), trevo (n = 1), and penumbra (n = 1). The time interval (minutes ± SD) between symptom onset and thrombus retrieval was 363 ± 238 minutes and between IV rt-PA initiation and thrombus retrieval was 170 ± 122 minutes. The time interval (minutes ± SD) between symptom onset and thrombus retrieval was similar in patients who received or did not receive IV rt-PA prior to endovascular treatment (326 ± 83 minutes vs. 386 ± 301 minutes).

The baseline and clinical characteristics of patients in whom thrombi were extracted is presented in Tables 1 and 2. The proportion of thrombi according to predominant composition was as follows: erythrocytes dominant (n = 7), fibrin dominant (n = 5), and mixed region dominant (n = 6). The time interval between symptom onset

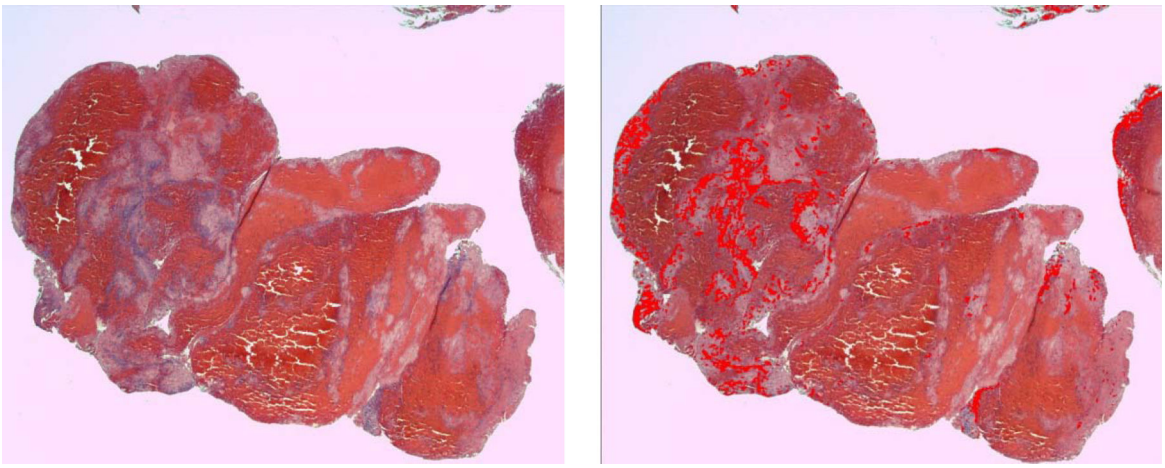

Figure 3. The basophilic cell aggregates in a rt-PA naïve thrombus (A) which is highlighted by automated thresholds to assign specific colors to clot component (B)

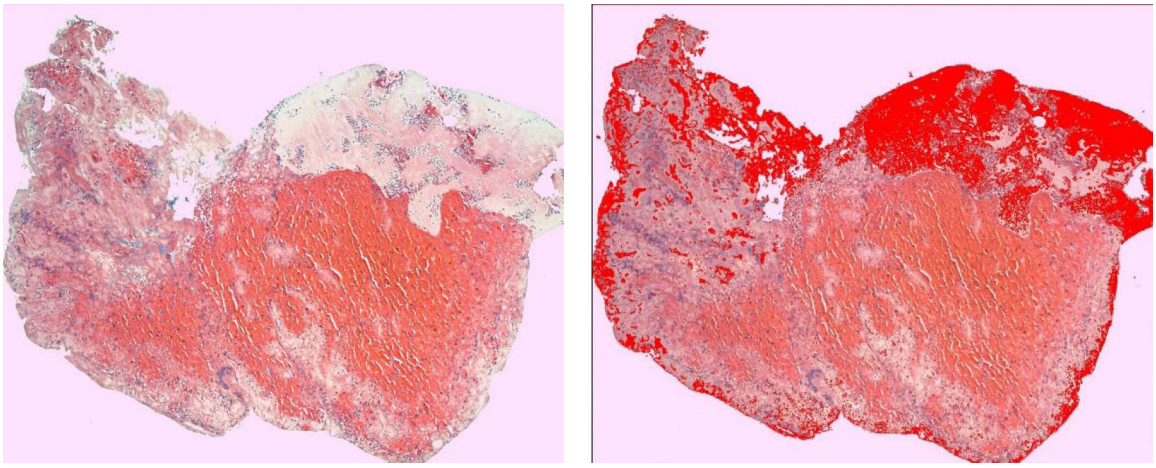

Figure 4. The fibrin deposition region in a rt-PA-resistant thrombus (A) which is highlighted by automated thresholds to assign specific colors to clot component (B)

and thrombus retrieval (mean ± SD) was 341 ± 106 min for erythrocytes dominant, 251 ± 67 minutes ± SD for fibrin dominant, and 482 ± 380 minutes for mixed region thrombi. The proportion of thrombi that were erythrocytes dominant was higher between those who did versus who did not receive IV rt-PA prior to endovascular treatment (57.1% vs. 27.3%). The proportion of mixed thrombi was lower in the patients who received or did not IV rt-PA (14.3% vs. 45.5%). The proportion of fibrin dominant thrombi was similar in those who did versus who did not receive IV rt-PA prior to endovascular treatment (28.6% vs. 27.3%). The erythrocytes aggregates were predominant topographic pattern in IV rt-PA-resistant thrombi compared with IV rt-PA naïve thrombi (57.1% vs. 36.4%). Random or repeated pattern of fibrin deposits with nest-like trapped erythrocytes clumps were more frequent in IV rt-PA naïve thrombi.

The quantitative analysis identified higher content of erythrocytes aggregates (39.2 ± 10.7% vs. 29.8 ± 15.5%) but lower eosinophilic amorphous material deposit content (21.2 ± 14.2% vs. 30.4 ± 23.4%) in IV rt-PA-resistant thrombi (Table 3). A time interval between symptom onset and thrombus retrieval ≥ 312 minutes was associated with lower content of eosinophilic amorphous material deposits (20.4 ± 14.5% vs. 33.3 ± 24.1%) but higher basophilic content (12.3 ± 5.5% vs. 8.3 ± 6.1%). In thrombi that were located in the middle cerebral artery, eosinophilic amorphous material deposit content was higher (32.5 ± 21.3% vs. 12.2 ± 5.1%) and mixed

Table 1. Clinical and thrombus characteristics of acute ischemic stroke patients with IV rt-PA resistant thrombi.

Age/gender	Time interval between symptom onset and thrombus retrieval (mins)	Initial NIHSS score	Location of occlusion	Cardiovascular risk factors	Thrombus type	Thrombus topography	Outcome at discharge (mRS)
80/man	383	19	Middle cerebral artery	Hypertension, hyperlipidemia	Erythrocytes dominant	Erythrocyte aggregates	1
63/man	427	17	Internal cerebral artery	Hyperlipidemia	Erythrocytes dominant	Random pattern	2
70/man	297	13	Middle cerebral artery	Hypertension, diabetes mellitus	Erythrocytes dominant	Erythrocyte aggregates	6
34/woman	424	31	Middle cerebral artery	Diabetes mellitus	Erythrocytes dominant	Erythrocyte aggregates	2
70/woman	253	20	Middle cerebral artery	Hypertension, hyperlipidemia	Mixed region dominant	Repeated pattern	5
76/woman	279	19	Middle cerebral artery	Hypertension, previous stroke	Fibrin dominant	Erythrocyte aggregates	6
79/man	225	19	Middle cerebral artery	None	Mixed region dominant	Repeated pattern	4

NIHSS – National institutes of Health stroke scale, mins – minutes, mRS-modified Rankin scale.

†Random or repeated pattern of fibrin deposits with nest-like trapped erythrocytes.

Table 2. Clinical and thrombus characteristics of acute ischemic stroke patients with IV rt-PA naïve thrombi.

Age/ gender	Time interval between symptom onset and thrombus retrieval (min)	Initial NIHSS score	Location of occlusion	Cardiovascular risk factors	Thrombus type	Thrombus topography	Outcome at discharge (mRS)
94/man	340	19	Middle cerebral artery	Hypertension, atrial fibrillation	Mixed region dominant	Erythrocyte aggregates	4
64/man	405	5	Middle cerebral artery	Hyperlipidemia, atrial fibrillation	Erythrocytes dominant	Erythrocyte aggregates	0
70/man	327	7	Vertebral artery	Hypertension, diabetes mellitus, hyperlipidemia, atrial fibrillation	Erythrocytes dominant	Random pattern†	5
64/woman	288	10	Middle cerebral artery	None	Mixed region dominant	Repeat pattern†	3
49/man	1213	2	Internal carotid artery	Hypertension, hyperlipidemia	Fibrin dominant	Repeat pattern†	4
93/woman	345	15	Middle cerebral artery	Hypertension	Fibrin dominant	Repeat pattern†	5
85/woman	245	14	Internal carotid artery	Hypertension, diabetes mellitus, hyperlipidemia	Mixed region dominant	Random pattern†	4
88/woman	586	10	Middle cerebral artery	Hypertension, hyperlipidemia, atrial fibrillation	Mixed region dominant	Random pattern†	2
68/man	126	18	Middle cerebral artery	Hypertension, hyperlipidemia, atrial fibrillation	Erythrocytes dominant	Random pattern†	4
79/woman	167	26	Middle cerebral artery	Hypertension, hyperlipidemia, atrial fibrillation	Fibrin dominant	Erythrocyte aggregates	6
49/man	211	15	Middle cerebral artery	Hypertension, hyperlipidemia	Fibrin dominant	Random pattern†	4

NIHSS – National institutes of Health stroke scale, mins – minutes, mRS-modified Rankin scale.

†Random or repeated pattern of fibrin deposits with nest-like trapped erythrocytes.

region content was less (24.3 ± 17.1% vs. 43.6 ± 10.1%) compared with thrombi in other locations.

Discussion

Previous studies have recognized the heterogeneity of thrombus composition in the analysis of thrombi retrieved from patients with acute ischemic stroke [11–15]. There are three patterns of morphology seen in patients with acute ischemic stroke: fibrin-dominant, erythrocytes-dominant, and mixed. The largest proportions of thrombi are fibrin-dominant in previous studies [11–15].

Our results confirm that majority of thrombi are mixed with variable content of erythrocytes and fibrin but the

Table 3. Proportion of thrombus components in various strata of patients.

	Erythrocytes aggregates (%) ± SD	Eosinophilic deposits (%) ± SD	Mixed region (%) ± SD	Basophilic component (%) ± SD
Patients with IV rt-PA naïve thrombi	29.8 ± 15.5	30.4 ± 23.4	30.3 ± 21.8	10.4 ± 6.6
Patients with IV rt-PA-resistant thrombi	39.2 ± 10.7	21.2 ± 14.2	28.5 ± 9.3	10.1 ± 5.3
Time interval between symptom onset and mechanical thrombectomy < 312[†] mins	31.4 ± 14.4	33.3 ± 24.1	28.8 ± 18.9	8.3 ± 6.1
Time interval between symptom onset and mechanical thrombectomy ≥ 312 mins	35.5 ± 14.7	20.4 ± 14.5	30.4 ± 17.3	12.3 ± 5.5
Location in middle cerebral artery	34.4 ± 16.0	32.5 ± 21.3	24.3 ± 17.1	9.9 ± 6.7
Location in other arteries	31.0 ± 9.8	12.2 ± 5.1	43.6 ± 10.1	11.4 ± 3.9
Premorbid use of aspirin	30.2 ± 11.5	27.6 ± 27.6	31.2 ± 19.0	11.0 ± 6.1
No premorbid use of aspirin	36.7 ± 16.7	26.1 ± 10.9	28.1 ± 17.1	9.6 ± 6.1

[†]The time interval is dichotomized based on median value.

rt-PA, recombinant tissue plasminogen activator

relative ratio of fibrin and erythrocytes was different in IV rt-PA-resistant thrombi (compared with IV rt-PA naïve thrombi). IV rt-PA-resistant thrombi appeared as large erythrocytes aggregates and higher content of erythrocytes on quantitative analysis. In contrast, IV rt-PA naïve thrombi were fibrin-dominant with erythrocytes nests within fibrin meshwork in repeated or random patterns. Higher content of eosinophilic deposits on quantitative analysis in such thrombi provided additional support for higher fibrin composition. There were some effect of time elapsed between symptom onset thrombus retrieval with both qualitative and quantitative analyses, each demonstrating higher erythrocytes content with increasing time. A higher content of basophilic cell content was apparent in thrombi acquired at later intervals from symptom onset. It should be noted that there was no exclusive pattern of thrombus in IV rt-PA-resistant thrombi and IV rt-PA naïve thrombi. Therefore, it is likely that secondary and ongoing thrombogenesis may shift thrombi toward relative homogenization.

There are two other observations that provide support to "high erythrocytes and low fibrin content" in thrombi that were resistant to IV rt-PA. Previous studies have demonstrated that hyperattenuation (or density) of thrombus is higher on CT scan with higher erythrocytes content [11,13]. Lower rates of recanalization are observed in patients with increased density within the artery subsequent to IV rt-PA administration [1,16]. Puig et al. [17] reported that the positive and negative predictive values for lack of recanalization after IV rt-PA for high-density thrombus on CT scan (rHU ≤ 1.382) were 93.75% and 100%, respectively. Within our own cohort, "high erythrocytes and low fibrin content" thrombi were located in arteries other than middle cerebral artery. Previous studies have consistently demonstrated lower rates of recanalization with thrombolytics in occlusions located in arteries other than middle cerebral artery [18,19].

The "relatively high erythrocytes and low fibrin content" of thrombi that were resistant to IV rt-PA is supported by previous observations that higher erythrocytes content in thrombi may lead to resistance to thrombolytics [20,21]. The erythrocytes triggered variability in the fibrin network structure, individual fiber characteristics, and overall thrombus viscoelasticity in one study [22]. Through the extension of projections, erythrocytes become intertwined within a thrombus to stabilize and strengthen its structure [20]. Erythrocytes confer lytic resistance to fibrin resulting from modified fibrin structure and impaired plasminogen activation [21]. Lines of Zahn were less frequently found in thrombi resistant to IV rt-PA. This feature is usually seen in thrombus formed at the site of rapid arterial blood flow, with laminations produced by successive deposition of platelets and fibrin (pale layers) alternating with erythrocytes (dark layers) [10]. Thrombus lysis by erythrocytes bound plasminogen activator begins in focal segments of the thrombus and takes longer for lysis process to complete compared with free plasminogen activators [23]. The other possibility is that thrombus composition was independent of IV rt-PA administration and related to the time interval between symptom onset and thrombus extraction. Simon et al [11] proposed that different morphological appearances are based on four phases of thrombus formation: erythrocytes dominant, erythrocytes proportion equal to fibrin, fibrin-dominant, and organized fibrin. There is a stepwise increase in fibrin content among thrombi retrieved from acute coronary occlusion based on the delay between symptom onset and retrieval [24]. In our cohort of patients, the relationship between time interval elapsed between symptom onset and thrombus retrieval and thrombus morphology did not support such an explanation. The time interval was shorter in patients with higher fibrin content compared with those with higher erythrocytes content thrombi. The possibility that "relatively high erythrocytes and low fibrin content" of thrombi may be a consequence of IV rt-PA exposure should be considered. It is possible that lysis and dissolution of fibrin induced by

IV rt-PA within an acute thrombus results in new aggregates of erythrocytes reforming the thrombus. The origin of the basophilic cells infiltrates within the thrombus is not well understood. The infiltrate may represent platelet aggregates within the thrombus. Platelets are either packed within fibrin-containing aggregates or seen as strings of nonaggregated platelets [25]. This inner zone of cytoplasm of the platelets contains bluish staining granules which are not individually visible and appear more or less homogeneously blue. The outer zone of platelet cytoplasm does not stain well enough for adequate visualization and visualization is further obscured by cellular contraction seen as part of thrombus retraction. The dense granules of platelets contain adenosine diphosphate (ADP) and ionized calcium, which are important contributors to thrombus formation. It is plausible that these cells represent basophilic granulocytes which have a two or three lobed nucleus that are trapped within the thrombus. The specific granules of basophils are stained deeply bluish or reddish-violet. The concentration in circulating blood of basophilic granulocytes is small and the only explanation for ≈8% thrombus content would be based on selective uptake of these cells into the thrombus. The granules within basophils contain heparin and histamine that may contribute to thrombus lysis. Basophilic stippling of erythrocytes may be a less plausible explanation secondary to degradation and abnormal collection of nucleic acid in dots throughout the cell.

There are some limitations that should be considered prior to the interpretation of our results. The sample size was small and any analysis assessing the relationship between thrombus compositions, angiographic recanalization, and clinical outcomes is subject to type II error. The heterogeneity of endovascular treatment in regards to administration of intravenous and/or intra- arterial thrombolytics and devices used adds an unmeasured bias on thrombus composition. However, such heterogeneity in endovascular treatment is consistent with current endovascular practices [26–31]. Retrieved thrombus may have undergone modifications during the retrieval process, such as loss of the intermediate zone, between the thrombus periphery and arterial wall [5]. Autopsy studies demonstrate well-organized thromboembolus and ingrowth of smooth muscle cells into the thromboembolus but are limited by the delay between acute stroke and observations of sample after death [32]. In another autopsy study, fibrin- and platelet-rich thrombi of various thicknesses develop at the atheromatous plaques of the cerebral arteries, followed by occlusion with fibrin- and red-cell-rich thrombi (red thrombi) [33]. The loss of peripheral zone and loosely attached components of thrombus is possible during the retrieval process. There may be components, such as endothelial cells present as isolated cells or clusters, that should not be necessarily components of initial thrombus but a secondary consequence of device retrieval [15]. We did not observe any calcification or endothelization as reported previously in anectdotal cases [34].

Although the observations are preliminary, we found "relatively high erythrocytes and low fibrin content" in thrombi that were resistant to IV rt-PA. It should be noted that there was no exclusive or pathognomic thrombus pattern seen in IV rt-PA-resistant thrombi and IV rt-PA naïve thrombi.

Conflict of Interest

The authors report that no conflicts of interest.

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