Thrombus Neutrophil Extracellular Traps Content Impair tPA-Induced Thrombolysis in Acute Ischemic Stroke

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Background and Purpose—Neutrophil Extracellular Traps (NETs) are DNA extracellular networks decorated with histones and granular proteins produced by activated neutrophils. NETs have been identified as major triggers and structural factors of thrombosis. A recent study designated extracellular DNA threads from NETs as a potential therapeutic target for improving tissue-type plasminogen activator (tPA)-induced thrombolysis in acute coronary syndrome. The aim of this study was to assess the presence of NETs in thrombi retrieved during endovascular therapy in patients with acute ischemic stroke (AIS) and their impact on tPA-induced thrombolysis.

Methods—We analyzed thrombi from 108 AIS patients treated with endovascular therapy. Thrombi were characterized by hematoxylin/eosin staining, immunostaining, and ex vivo enzymatic assay. Additionally, we assessed ex vivo the impact of deoxyribonuclease 1 (DNAse 1) on thrombolysis of AIS thrombi.

Results—Histological analysis revealed that NETs contributed to the composition of all AIS thrombi especially in their outer layers. Quantitative measurement of thrombus NETs content was not associated with clinical outcome or AIS pathogenesis but correlated significantly with endovascular therapy procedure length and device number of passes. Ex vivo, recombinant DNAse 1 accelerated tPA-induced thrombolysis, whereas DNAse 1 alone was ineffective.

Conclusions—This study suggests that thrombus NETs content may be responsible for reperfusion resistance, including mechanical or pharmacological approaches with intravenous tPA, irrespectively of their etiology. The efficacy of a strategy involving an administration of DNAse 1 in addition to tPA should be explored in the setting of AIS.

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Key Words: extracellular traps ■ fibrinolysis ■ neutrophils ■ stroke ■ thrombosis

Intravenous administration of tissue-type plasminogen activator (tPA) is the unique pharmacological therapy recommended in acute ischemic stroke (AIS) patients. This treatment has a low efficiency in terms of early recanalization, especially in the setting of large vessel occlusion.[1] Although intravenous tPA combined with endovascular therapy (EVT) is currently the standard of care to improve 3-month functional outcome in AIS patients with large vessel occlusion,[2] the relevance of intravenous tPA on top of EVT is debated. EVT has opened up a new field of research, making possible the analysis of thrombi responsible for intracranial occlusion, and in particular, intravenous tPA-resistant ones. Neutrophil extracellular traps (NETs) have recently been identified as key players involved in the formation of thrombi of various origins.[3] NETs are fibrous networks of extracellular DNA released by neutrophils under the form of decondensed chromatin associated with histones and neutrophil granule proteins such as myeloperoxidase and neutrophil elastase.[4] A recent study showed that coronary thrombus NETs content was associated with poor outcome in myocardial infarction and identified NETs as a potential therapeutic target to improve tPA-induced thrombolysis efficacy.[5]

The aim of the present study was to assess the presence of NETs in thrombi retrieved from patients with AIS during EVT and to evaluate the association with AIS pathogenesis, EVT procedure modalities, and clinical outcome. A secondary objective was to investigate if targeting NETs with recombinant deoxyribonuclease 1 (DNAse 1) could improve tPA-induced thrombolysis.

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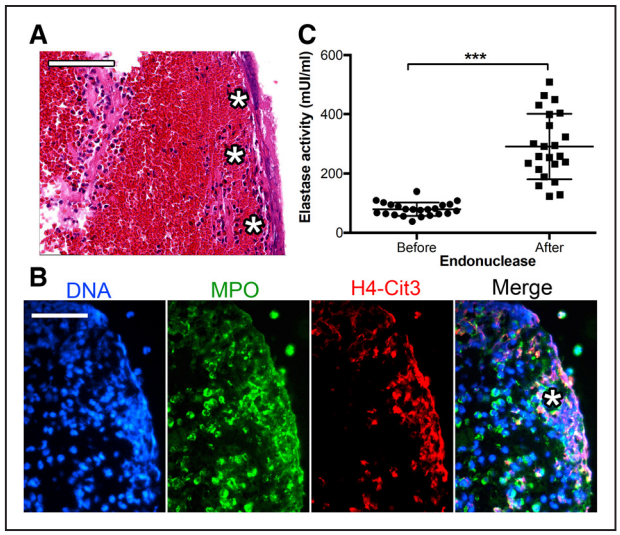

Figure 1. Neutrophil extracellular traps (NETs) are constitutively present in acute ischemic stroke thrombi. The presence of neutrophils and NETs in acute ischemic stroke (AIS) thrombi was investigated by immunohistological analysis. **A**, Representative image of a thrombus stained by hematoxylin/eosin showing the abundance of polymorphonuclear cells and extracellular nucleic acid (*) predominantly located in the thrombus outer layer. Scale bar=50 μm. **B**, Representative images of a thrombus stained for DNA (DAPI; Sigma-Aldrich), and with antibodies against myeloperoxidase (rabbit antihuman MPO antibody, Dako) and citrullinated histone H4 (rabbit antihuman Histone H4 citrulline 3 [H4-Cit3] antibody, Millipore). Note the presence of NETs at the thrombus periphery. Scale bar=50 μm. **C**, The presence of NETs in AIS thrombi was investigated by measurement of DNA-associated neutrophil elastase activity. The dot plot shows the elastase activity measured in supernatants of thrombi before and after endonuclease treatment (n=23; $P<0.0001$).

Methods

The authors declare that all supporting data are available within the article and in the online-only Data Supplement.

Patients treated in Rothschild Foundation Hospital by EVT with successful thrombus retrieval were enrolled in this study. The study protocol was approved by the Comité de Protection des Personnes Ile-de-France VI. This local institutional review board waived the need for patient written informed consent.

Thrombi were characterized by hematoxylin/eosin staining, immunostaining, ex vivo NETs assessment, and ex vivo thrombus lysis assay. Detailed materials and methods are available in the online-only Data Supplement.

Results

Patient's Characteristics

We included 108 patients between December 2015 and December 2016 in this study. Patient characteristics are listed in the Table I in the online-only Data Supplement. These characteristics are similar to those of recently published EVT trials.

NETs Are Constitutively Present in AIS Thrombi

The morphometric analysis of AIS thrombi after hematoxylin/eosin staining revealed the presence of numerous polymorphonuclear cells in all thrombi examined. Strands of extracellular nucleic acid suggesting of NETs were found in all 34 thrombi analyzed in histology, especially in their superficial layers (Figure 1A).

Immunofluorescence detection confirmed that areas containing extracellular DNA colocalized with citrullinated histones and granular neutrophils proteins (myeloperoxidase), which correspond to NETs (Figure 1B; Figure I in the online-only Data Supplement). To confirm the presence of NETs in AIS thrombi, we realized an ex vivo NETs assay in 23 thrombi. For this assay, NETs presence was investigated by measuring neutrophil elastase activity released from thrombi after endonuclease treatment. Neutrophil elastase activity was increased after endonuclease incubation, thus confirming the presence of NETs in all thrombi (Figure 1C).

Neutrophil-Derived Extracellular DNA Content Correlates With EVT Procedure Length and Device Number of Passes

Thrombus neutrophil-derived extracellular DNA content was quantified by measuring neutrophil elastase antigen released

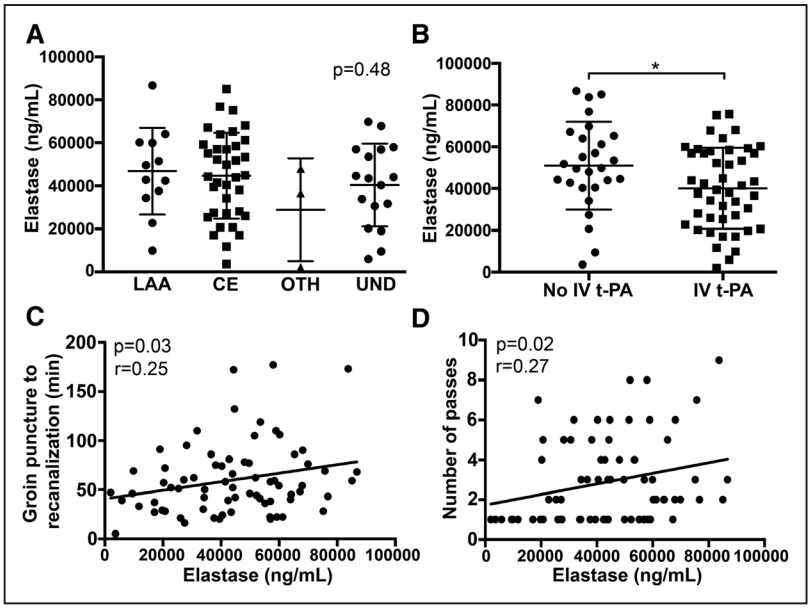

Figure 2. Thrombus neutrophil-derived extracellular DNA content is not associated with acute ischemic stroke (AIS) pathogenesis but with endovascular procedure characteristics. **A** and **B**, Comparison of neutrophil-derived extracellular DNA content (n=72) between thrombi classified according to (**A**) stroke pathogenesis (LAA, atherosclerosis; CE, cardioembolic; OTH, other cause; UND, undetermined) or (**B**) to administration or not of intravenous tPA treatment before endovascular therapy ($P=0.03$). **C** and **D**, Correlation between thrombus neutrophil-derived extracellular DNA content (n=72) and endovascular procedure length (**C**); and number of device passes achieved (**D**). IV indicates intravenous.

from thrombi treated with endonuclease (n=72). There was no significant correlation between NETs content and stroke pathogenesis, 3-month functional outcome or final Thrombolysis in Cerebral Infarction (TICI) score (Figure 2A; Figure II in the online-only Data Supplement). NETs thrombus content from patients previously treated with intravenous tPA was significantly reduced compared with thrombi from patients without intravenous tPA therapy (Figure 2B). Finally, NETs content was positively correlated with endovascular procedure length and device number of passes (Figure 2C and 2D).

Targeting NETs With DNAse 1 Accelerated Ex Vivo tPA-Induced Thrombolysis

To test whether NETs targeting with DNAse 1 could enhance thrombolysis, we performed an ex vivo lysis assay in 24 AIS thrombi. In a first experiment, we compared tPA alone versus tPA+DNAse 1 (n=13). The addition of DNAse 1 to tPA significantly accelerated ex vivo thrombolysis. In a second experiment, we compared DNAse 1 alone versus tPA+DNAse 1 (n=11). DNAse 1 alone was ineffective to induce a significant thrombolysis (Figure 3A and 3B).

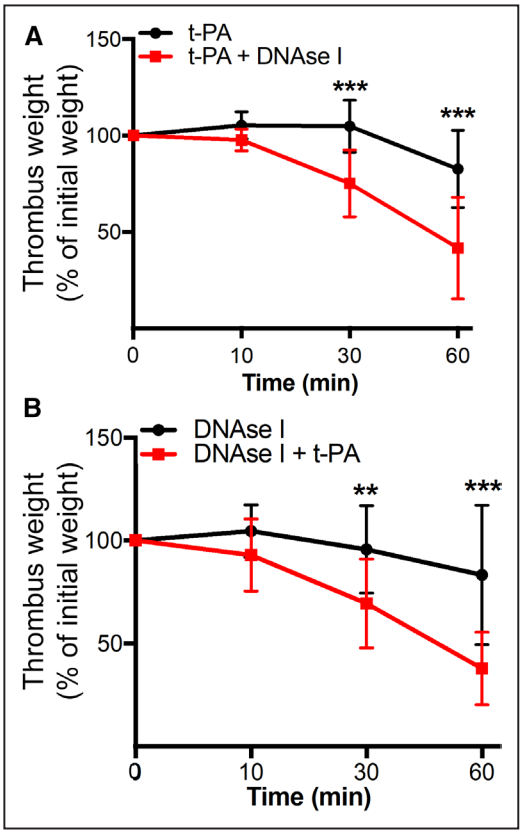

Figure 3. DNAse 1 potentiates tPA-induced thrombolysis ex vivo. Acute ischemic stroke thrombi recovered by endovascular therapy were incubated with tPA and DNAse 1, and their lysis was followed by measurement of thrombus wet-weight evolution over time. Mean baseline weight of thrombi was 14, 6±8, and 4 mg. **A**, Comparison of the thrombolytic effect of tPA alone or in combination with DNAse 1 (n=13, mean [SD]; 10 min, tPA=105.3% [7.02] vs tPA+DNAse 1=97.73% [5.62], [*P*=0.022]; 30 min, tPA=104.8% [13.45] vs tPA+DNAse 1=75.13% [17.39], [*P*=0.001]; 60 min, tPA=82.71% [20.08] vs tPA+DNAse 1=41.71% [26.43], [*P*=0.007]). tPA alone is associated with a slightly but significant thrombus weight reduction at 60 min compared with baseline (*P*=0.003). **B**, Comparison of the thrombolytic effect of DNAse 1 alone or in combination with tPA (n=11, mean [SD]; 10 min, DNAse 1=104.6% [12.73] vs DNAse 1+tPA=92.91% [17.55]; 30 min: DNAse 1=95.66% [21.29] vs DNAse+tPA=69.39% [21.65]; 60 min: DNAse 1=83.36% [33.89] vs DNAse 1+tPA=37.83% [17.65]). DNAse 1 alone has no impact on thrombus weight after 60 min compared with baseline (*P*=0.06).

Discussion

Our study shows that (1) all AIS thrombi contain NETs irrespectively of the stroke pathogenesis, (2) NETs are predominantly located in the outer layer of AIS thrombi, (3) NETs content is associated with endovascular procedure length and device number of passes, and (4) tPA and DNAse 1 coadministration accelerates ex vivo thrombolysis compared with tPA or DNAse 1 alone.

Our findings are in line with a recent report showing that NETs are important constituents of AIS thrombi.[6] The latter study found a significant higher amount of NETs in AIS thrombi from cardiac origin compared with noncardiac thrombi. In the present study, we do not find a correlation between NETs and stroke pathogenesis but, maybe more importantly, show that NETs contribute to the scaffold of thrombi irrespectively of their origin. This particular architecture may participate in tPA resistance, as suggested by a dramatic increase in ex vivo tPA-induced thrombolysis in the presence of DNAse 1. Our results support recent evidence indicating that DNAse 1 could help to potentiate tPA-induced lysis of human coronary and AIS thrombi.[5,6] Notably, we show here that treatment with DNAse 1 alone has no thrombolytic effect ex vivo, which indicates that both fibrin and neutrophil-derived extracellular DNA matrix have to be targeted to induce successful thrombolysis.

A previous study has shown that extracellular DNA and histones do modify the structure of fibrin, rendering it more resistant to mechanical and enzymatic destruction.[7] This fact may explain the observed correlation between NETs content and the number of device passes performed to achieve a successful recanalization. In this perspective, NETs may participate in the interaction between the thrombus and the arterial wall or between the thrombus and the EVT device, thus increasing the difficulty for a successful thrombus removal during EVT.

Interestingly, previous experimental studies have already assessed the impact of DNAse 1 infusion in models of ischemia–reperfusion. In a mouse model of cerebral ischemia–reperfusion, DNAse 1 alone was found to significantly reduce the infarct volume compared with vehicle.[8] In a myocardial ischemia–reperfusion model in rats, DNAse 1 alone was ineffective but DNAse 1 and tPA coadministration reduced significantly the infarct size.[9] These discordant results may be explained by the endogenous expression of tPA in brain capillaries, which would be in favor of an effect of DNAse 1 alone in brain.[10] Therefore, DNAse 1 administration on top on intravenous tPA could have a favorable impact on proximal arterial recanalization rate but also in downstream microvascular thrombosis.

Further optimization of thrombolysis might come from evaluation of the endothelial contribution to tPA resistance. For instance, the protein C pathway plays a crucial role in coagulation and inflammation. In fact, previous studies have shown that elevated soluble endothelial protein C receptor are

associated with thrombolysis resistance in AIS patients with large vessel occlusion.[11]

Finally, these evidence support a pharmacological cocktail for the future of AIS treatment including therapies targeting the thrombus embedded in the large vessel, the activated endothelium, and the downstream microvascular thrombosis.[12]

Conclusions

NETs form a scaffold responsible, at least in part, for thrombus tPA resistance. Our data support the concept that DNAse 1 infusion could have a synergistic action to improve the efficacy of intravenous tPA-induced thrombolysis in AIS.

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Disclosures

None.

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