

Altered Fibrin Clot Structure/Function in Patients With Cryptogenic Ischemic Stroke

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Background and Purpose—We tested the hypothesis that fibrin structure/function is unfavorably altered in patients with cryptogenic ischemic stroke.

Methods—Ex vivo plasma fibrin clot permeability, turbidimetry, and efficiency of fibrinolysis were determined in 89 patients with patent foramen ovale (PFO) and a history of first-ever stroke, 58 patients with first-ever stroke and no PFO, and 120 healthy controls.

Results—Stroke patients, evaluated 3 to 19 months after the event, and controls did not differ with regard to age, sex, smoking, and fibrinogen. Stroke patients with or without PFO had lower clot permeability ($P<0.0001$), faster fibrin polymerization ($P<0.0001$), prolonged clot lysis time ($P<0.0001$), higher maximum D-dimer levels released from clots ($P<0.0001$), and maximum rate of D-dimer release ($P=0.02$) than controls. Time from stroke occurrence showed no association with any clot variables. Scanning electron microscopy of fibrin clots showed increased fiber diameter and density in stroke patients. Clots from stroke patients with PFO were more permeable and showed shorter lysis time compared to those without PFO, and this was related to lower proportion of smokers in the former group.

Conclusions—Altered fibrin clot structure and resistance to fibrinolysis are associated with cryptogenic stroke. (*Stroke*. 2009;40:1499-1501.)

Key Words: fibrin clot ■ fibrinolysis ■ stroke

Strokes without a definite cause (cryptogenic) after extensive workup constitute $\approx 30\%$ to 40% of all strokes.¹ Growing evidence indicates that patent foramen ovale (PFO) is frequently observed in patients with cryptogenic stroke.²

Blood coagulation leads ultimately to the formation of a fibrin clot that is preceded by fibrinogen conversion to fibrin and fibrin cross-linking. Fibrin clot properties are influenced by environmental and genetic factors, especially fibrinogen concentrations.³ We hypothesized that altered fibrin clot structure/function is associated with cryptogenic stroke. The rationale for this hypothesis is based on the recognition that formation of compact fibrin clots resistant to fibrinolysis predisposes to thrombotic events.³

The aim of this study was to investigate fibrin clot structure and function in patients with cryptogenic stroke associated with or without PFO.

Subjects and Methods

We studied white consecutive subjects aged younger than 65 years, including 89 patients with PFO and a history of first-ever ischemic stroke, including 80 after successful transcatheter PFO closure 4 to

17 months before the study, and 58 patients with previous first-ever ischemic stroke and without PFO. Stroke was diagnosed based on the occurrence of a new and abrupt focal neurological deficit persisting for >24 hours and confirmed by positive findings in CT or MRI. All strokes were cryptogenic according to the TOAST criteria despite a thorough evaluation, including carotid ultrasound, echocardiography, Holter monitoring, and autoimmune work-up. Exclusion criteria were any acute illness, inflammatory states (C-reactive protein >10 mg/L), cancer, atrial fibrillation, diabetes, hepatic or renal dysfunction, thienopyridine or anticoagulant therapy, valvular heart disease, symptomatic coronary artery disease, the common or internal carotid artery stenosis ($\geq 30\%$ diameter), and recurrent or lacunar strokes.

All patients underwent transthoracic and transesophageal echocardiography. PFO was diagnosed with injections of agitated saline at rest and with Valsalva maneuver.² Atrial septal aneurysm defined as described² was found in 66% of PFO subjects. One hundred twenty apparently healthy white individuals served as controls. They were free from cardiovascular events and had normal echocardiography. The University Ethical Committee approved the study.

Laboratory Investigations

Lipid profiles, blood cell count, glucose, creatinine, fibrinogen, and C-reactive protein were measured in fasting blood. We determined in plasma prothrombin fragments 1.2 (F1.2), a marker of thrombin formation (Dade Behring); D-dimer, a marker of fibrin turnover

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Table 1. Characteristics of Stroke Patients and Healthy Controls

	Stroke Patients (n=147)	Stroke Patients With PFO (n=89)	Stroke Patients Without PFO (n=58)	Controls (n=120)
Age, yr, male, n (%)	44.2±14.5 45 (30.6)	42.7±14.1 26 (29.2)	46.0±14.8 19 (32.8)	44.8±12.4 40 (33.3)
Current smokers, n (%)	45 (30.6)	18 (20.2)*	27 (46.6)†	41 (34.1)
Hypertension, n (%)	57 (38.8)*	30 (33.7)*	27 (46.5)*	0
Aspirin, n (%)	138 (93.9)*	80 (89.9)*	58 (100)*	0
Statins, n (%)	49 (33.3)*	28 (31.5)*	21 (36.2)*	0
β-blockers, n (%)	52 (35.4)*	28 (31.5)*	24 (41.4)*	0
ACE inhibitors, n (%)	49 (33.3)*	13 (28.9)*	36 (62.1)*†	0
Fibrinogen, g/L	3.19±0.96	3.28±0.95	3.0±0.96	2.98±0.82
CRP, mg/L	1.81±1.35	2.09±1.60	1.37±0.64†	1.61±0.97
D-dimer, mg/dL	180.2±81.3	195.2±81.0	157.1±76.9	166.4±48.4
tPA, ng/mL	9.82±3.17*	9.50±3.27	10.30±2.98*	8.58±3.17
PAI-1, ng/mL	14.38±6.63*	13.64±6.53	15.51±6.68*	12.04±4.46
F1.2, nmol/L	0.72±0.18	0.78±0.20	0.71±0.15	0.73±0.20

ACE indicates angiotensin-converting enzyme; CRP, C-reactive protein; PAI-1, plasminogen activator inhibitor-1; tPA, tissue plasminogen activator.

* $P<0.05$ compared to controls.

† $P<0.01$ compared to stroke patients with PFO.

(American Diagnostica); antigens of tissue-type plasminogen activator; and plasminogen activator inhibitor-1 (American Diagnostica).

Fibrin Clot Properties

In citrated plasma (vol/vol, 9:1 of 3.2% sodium citrate), the following variables were determined at least in duplicates by technicians blinded to the origin of the samples (intraassay and interassay coefficients of variation, 5% to 8%), as previously described in detail.^{4–6}

1. Fibrin clot permeation using a pressure-driven system, with calculation of a permeation coefficient (K_s), which indicates the pore size.^{5,7} Lower K_s values indicate reduced permeability.
2. The lag phase of the turbidity curve, which reflects the time required for initial protofibril formation and maximum absorbance at 405 nm at the plateau phase (Δ Abs), indicating the number of protofibrils per fiber.^{4,7,8}
3. Clot lysis time, defined as the time required for a 50% decrease in clot turbidity at 405 nm ($t_{50\%}$), induced by 1 μ g/mL recombinant tissue plasminogen activator (Boehringer Ingelheim).^{4,6,9}
4. Maximum rate of increase in D-dimer levels (American Diagnostica) and maximum D-dimer concentrations measured every 30 minutes in a buffer containing 0.2 μ mol/L recombinant tissue plasminogen activator (Boehringer Ingelheim) percolating through fibrin clots formed as for permeation evaluation.^{4,5,10}

Table 2. Fibrin Clot Variables

	Stroke Patients (n=147)	Stroke Patients With PFO (n=89)	Stroke Patients Without PFO (n=58)	Controls (n=120)
K_s , 10^{-9} cm ²	6.98±1.62*	7.50±1.62*	6.20±1.27*†	9.06±1.56†
Lag phase, sec	38.14±3.78*	37.63±3.58*	38.91±3.97*	44.89±4.25†
Δ Abs (405 nm)	0.90±0.11*	0.91±0.11*	0.90±0.12*	0.73±0.07†
$t_{50\%}$, min	9.39±1.31*	9.14±1.25*	9.77±1.31*†	7.80±1.08†
D-D max, mg/L	4.11±0.56*	4.09±0.56*	4.14±0.57*	3.58±0.44†
D-D rate, mg/L/min	0.074±0.008*	0.075±0.008*	0.073±0.008	0.072±0.009†

Δ Abs (405 nm) indicates maximum absorbance of fibrin gel at 405 nm in turbidimetry; D-D max, maximum D-dimer levels in the lysis assay; D-D rate, maximum rate of increase in D-dimer levels in the lysis assay; K_s , permeability coefficient; $t_{50\%}$, lysis time.

* $P<0.05$ compared to controls.

† $P<0.01$ compared to stroke patients with PFO.

Scanning Electron Microscopy

Clots from 5 stroke patients and 5 controls with similar fibrinogen levels were fixed by permeating them with a 2.5% glutaraldehyde solution for 2 hours, recovered from the tubes, and further processed by dehydration.^{5,7} Samples were photographed digitally in 5 different areas with a Hitachi S-4700 SEM. Fiber thickness was measured as described.^{5,11}

Statistical Analysis

Data were given as mean±SD or otherwise stated. The significance of between-group differences was tested by analysis of variance with Scheffe adjustment. Categorical values were analyzed using the χ^2 test or Fisher exact test. The Mann-Whitney U test or Student t test was used to test differences between 2 groups as appropriate. Post hoc comparisons were made using Tukey test. Correlations were assessed by the Spearman test. A value of $P<0.05$ was considered significant.

Results

Stroke Patients vs Healthy Controls

A total of 147 patients who experienced stroke at a median of 8 (3–19) months before enrollment were studied. Table 1 shows characteristics of the patients and controls. Most patients were using low-dose aspirin (8–22 months). Lipid profile, glucose, and creatinine were similar in both groups (data not shown).

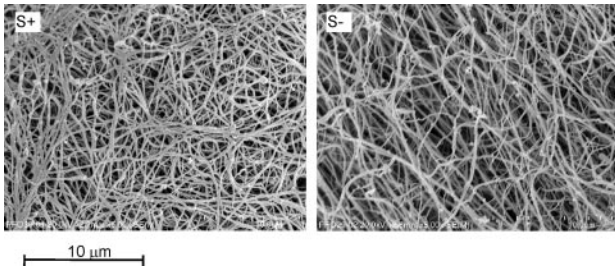


Figure. Representative scanning electron microscopy images from 1 stroke patient (S+) and 1 stroke-free subject (S-) with similar fibrinogen concentrations. The S+ demonstrates tight fibrin network and increased fiber diameter compared with the S-.

Only tissue plasminogen activator and plasminogen activator inhibitor-1 antigen levels were slightly higher in the patients.

Stroke patients had lower clot permeability, longer lysis time, a shorter lag phase, and greater maximum Δ Abs (Table 2). Time-courses of the D-dimer release from clots demonstrated that maximum rates of fibrinolysis and maximum D-dimer levels in the effluent were higher in the patients (Table 2). No differences in clot features associated with gender, smoking, hypertension, medication, or the time that elapsed from the ischemic event were observed. Overall fiber diameter and an average number of fibrin fibers were greater in stroke patients than stroke-free subjects (169 [157–180] vs 148 [145–150] nm; $P=0.009$; and 14.8 [14.7–14.8] vs 11.4 [11.0–12.1]; $P=0.01$; respectively; Figure).

Stroke Patients With and Without PFO

While comparing stroke patients with and without PFO (Table 1), we found fewer smokers and angiotensin-converting enzyme inhibitor-treated patients, and higher C-reactive protein in the former group.

Clots made from plasma of stroke patients with PFO were more permeable and showed shorter lysis time compared to stroke patients without PFO, without any intergroup differences in other clot parameters (Table 2). There was an association between lower percentage of smokers and more favorable clot features in stroke patients with PFO ($P=0.02$). None of the clot variables correlated with the time from the PFO closure or stroke occurrence.

Discussion

This study demonstrates that altered clot properties are associated with cryptogenic stroke. In stroke patients, both with and without PFO, fibrin clots were formed more rapidly and had a compact structure composed of thicker fibers compared to those made from plasma obtained from healthy controls. Compact clot structure results in impaired transport of proteins through networks and regardless of fiber thickness is associated with slow fibrinolysis,³ as shown also in this study.

Mechanisms behind altered clot structure/function in stroke patients are not clear. Fibrinogen is a major predictor of fibrin clot properties.^{3,8} Of note, differences between stroke patients and controls in these properties were observed despite similar fibrinogen levels. Fibrin clot properties are likely even worse in stroke patients not receiving aspirin that increases clot permeability and facilitates fibrin lysis.^{3,9} Alterations in fibrin function/structure in stroke patients with

PFO cannot be attributed to the activation of coagulation observed shortly after PFO closure.¹¹ Higher clot permeability and faster lysis in stroke patients with PFO than in those without PFO is likely explained by lower proportion of smokers in the former group. Smoking is known to unfavorably alter clot properties.³ We cannot exclude that lipoprotein(a) or homocysteine affecting clot properties,^{3,6,12} or genetic factors, not measured in the present study, might be implicated in stroke-related clot abnormalities.

Our study has limitations. The number of study participants was limited. We determined variables at a single time point. Exclusion of subjects with multiple strokes as well other than cryptogenic strokes resulted in uncertainty whether our findings refer also to these patients. Our stroke patients with PFO had atrial septal aneurysm in 66%; therefore, it is unclear whether our results can be extrapolated to other PFO subjects. Finally, statistical associations do not necessarily mean the cause–effect relationship and further studies are needed.

In conclusion, patients with cryptogenic stroke, both with and without PFO, are characterized by formation of dense fibrin clots resistant to lysis. Our study provides new insights into the pathophysiology of ischemic stroke that might have practical implications.

Disclosures

None.

References

- Guercini F, Acciarresi M, Agnelli G, Paciaroni M. Cryptogenic stroke: time to determine aetiology. *J Thromb Haemost.* 2008;6:549–554.
- Hara H, Virmani R, Ladich E, Mackey-Bojack S, Titus J, Reisman M, Gray W, Nakamura M, Mooney M, Poulouse A, Schwartz GL. Patent foramen ovale: current pathology, pathophysiology, and clinical status. *J Am Coll Cardiol.* 2005;46:1768–1776.
- Scott EM, Ariens RAS, Grant PJ. Genetic and environmental determinants of fibrin structure and function. Relevance to clinical disease. *Arterioscler Thromb Vasc Biol.* 2004;24:1558–1566.
- Undas A, Celinska-Lowenhoff M, Lowenhoff T, Szczeklik A. Statins, fenofibrate, and quinapril increase clot permeability and enhance fibrinolysis in patients with coronary artery disease. *J Thromb Haemost.* 2006;4:1029–1036.
- Undas A, Szuldrzynski K, Stepień E, Zalewski J, Godlewski J, Tracz W, Pasowicz M, Zmudka K. Reduced clot permeability and susceptibility to lysis in patients with acute coronary syndrome: effects of inflammation and oxidative stress. *Atherosclerosis.* 2007;196:551–558.
- Undas A, Brozek J, Jankowski M, Siudak Z, Szczeklik A, Jakubowski H. Plasma homocysteine affects fibrin clot permeability and resistance to lysis in human subjects. *Arterioscler Thromb Vasc Biol.* 2006;26:1397–1404.
- Mills JD, Ariens RA, Mansfield MW, Grant PJ. Altered fibrin clot structure in the healthy relatives of patients with premature coronary artery disease. *Circulation.* 2002;106:1938–1942.
- Blombäck B, Carlsson K, Hessel B, Liljeborg A, Procyk R, Aslund N. Native fibrin gel networks observed by 3D microscopy, permeation and turbidity. *Biochim Biophys Acta.* 1989;997:96–110.
- Williams S, Fatah K, Ivert T, Blombäck M. The effect of acetyl salicylic acid on fibrin gel lysis by tissue plasminogen activator. *Blood Coagul Fibrinolysis.* 1995;6:718–725.
- Collet J-P, Mishal Z, Lesty C, Mirshahi M, Payne J, Baumelou A, Bensman A, Soria J, Soria C. Abnormal fibrin clot architecture in nephritic patients is related to hypofibrinolysis: influence of plasma biochemical modifications. *Thromb Haemost.* 1999;82:1482–1489.
- Bedard E, Rodes-Cabau J, Houde C, Mackey A, Rivest D, Cloutier S, Noel M, Marre A, Cote JM, Chetaille P, Delisle G, Leblanc MH, Bertrand OF. Enhanced thrombogenesis but not platelet activation is associated with transcatheter closure of patent foramen ovale in patients with cryptogenic stroke. *Stroke.* 2007;38:100–104.
- Undas A, Stepień E, Tracz W, Szczeklik A. Lipoprotein(a) as a modifier of fibrin clot permeability and susceptibility to lysis. *J Thromb Haemost.* 2006;4:973–975.