Endothelial Activation in Lacunar Stroke Subtypes

Iris L.H. Knottnerus, MD; Jose W.P. Govers-Riemslag, PhD; Karly Hamulyak, MD, PhD; Rob P.W. Rouhl, MD; Julie Staals, MD, PhD; Henri M.H. Spronk, PhD; Rene van Oerle; Elisabeth P.M. van Raak, MD, PhD; Jan Lodder, MD, PhD; Hugo ten Cate, MD, PhD; Robert J. van Oostenbrugge, MD, PhD

Background and Purpose—Lacunar stroke (LS) can be subtyped according to the absence (isolated lacunar infarct [ILA]) or presence of concomitant white matter lesions (WML) and/or asymptomatic lacunar infarcts. Endothelial activation is thought to play a pivotal role in the subtype with WML and/or asymptomatic lacunar infarcts. The aim of this study was to evaluate whether endothelial activation is associated with WML and/or asymptomatic lacunar infarcts in LS patients. Here, we determined levels of circulating blood markers of endothelial function in LS patients.

Methods—In 149 patients, all of whom had brain-MRI, levels of tissue plasminogen activator (tPA), plasminogen activator inhibitor type 1 (PAI-1), tPA–PAI-1 complex, von Willebrand factor, thrombomodulin, and coagulation factor VIII were determined. Levels of blood markers were related to subtypes of LS and adjusted for age, gender, and vascular risk factors.

Results—In subtypes of LS, tPA activity was increased in patients with WML (0.79 IU/mL vs 0.44 IU/mL for ILA; P=0.02) and PAI-1-antigen levels were lowest in patients with WML (27.5 ng/mL vs 44.0 ng/mL for ILA; P=0.02). The association between WML and PAI-1 remained significant after multivariable analysis (OR, 0.99; 95% CI, 0.98–1.00 per ng/mL change of PAI-1; P=0.04).

Conclusions—We found further evidence for the hypothesis of endothelial activation in the subtype of LS caused by a diffuse small vessel vasculopathy, as we found higher levels of tPA in patients with concomitant extensive WML than in those with ILA. Second, low levels of PAI-1 were associated with WML. We postulate that differences in activity of components of the fibrinolytic system might contribute to WML development. (Stroke. 2010;41:1617-1622.)

Key Words: endothelial dysfunction ■ endothelium ■ lacunar stroke

Lacunar stroke accounts for one-quarter of all ischemic strokes and is characterized by one of the lacunar syndromes with a compatible lacunar infarct on brain imaging. Lacunar infarcts are located deep within the brain and caused by the occlusion of a single perforating artery. Several pathology and clinical studies support the hypothesis that there are different forms of lacunar stroke, ie, isolated lacunar infarct (ILA) caused by a small atheromatous plaque and lacunar stroke with concomitant diffuse white matter lesions (WML) and asymptomatic lacunar infarcts (aLAC), caused by a generalized diffuse destruction of the vessel wall.

Recent observations suggest that activation of the cerebral microvascular endothelium might be the primary step in the pathogenesis of lacunar stroke, especially in the subtype with concomitant WML and aLAC. Activation of the endothelium leads to increased permeability of the blood–brain barrier, which allows plasma components to reach the perivascular space and cause glial and neuronal damage. The function of vascular endothelial cells can be assessed in vivo by measuring levels of circulating molecules of endothelial origin. Because of its hemostatic barrier function, a number of endothelium-derived proteins are typically related to hemostasis and coagulation. The von Willebrand factor (vWF) connects the blood platelet to the subendothelial collagen. In plasma, it circulates in a noncovalent complex with coagulation factor VIII. Tissue factor (TF) is normally only encountered by the blood in case of endothelial disruption, but a wide variety of agents can lead to expression of TF on cultured endothelial cells. By binding to activated factor VIIa, TF initiates the extrinsic coagulation pathway. Thrombomodulin (TM) is normally expressed on the endothelial cell surface, where it mediates the activation of the anticoagulant protein C. Tissue plasminogen activator (tPA) converts plasminogen to plasmin, which lyses clots. The activity of tPA is inhibited by binding to plasminogen...
activator inhibitor type 1 (PAI-1), which is also constitutively secreted by the endothelium.15

The aim of this study was to determine whether endothelial activation was associated with WML and/or aLAC. Here, we determined the levels of circulating blood markers of endothelial function in lacunar stroke patients and expected to find the highest levels of these markers in the subtype with concomitant WML and/or aLAC as compared to ILA.

Subjects and Methods

Study Population

From May 2003 until December 2007, all residential consecutive patients presenting at emergency department or outpatient clinic with a first-ever ischemic stroke (n=1093) were registered in the Maastricht Stroke Registry.5 Lacunar stroke was defined as one of the recognized lacunar syndromes with a lesion on imaging compatible with the occlusion of a single perforating artery or, if no such lesion was visible on imaging, using established criteria of unilateral motor and/or sensory signs involving the whole of at least 2 of the 3 body parts (face, arm, and leg) without disturbance of consciousness or cortical functions.1 To increase likelihood that the lacunar syndrome was visible on imaging, using established criteria of unilateral motor disease (at least 1 internal carotid artery with 50% stenosis). Of the remaining lacunar stroke patients (n=234), 117 did not participate for several reasons (eg, contraindication for MR imaging, use of oral anticoagulants, heparin or low-molecular-weight heparin, extensive comorbidity, unwilling to participate in scientific research). Participating patients were younger and more often male than those who refused to participate.

Applying the same criteria, we also recruited 32 lacunar stroke patients from a nearby hospital (Orbis Medical Center; Sittard, the Netherlands). Age and gender, as well as vascular risk factor profile, were recorded. Hypertension was defined as repeated measurements of systolic blood pressure >140 mm Hg and diastolic blood pressure >90 mm Hg or current treatment with antihypertensive drugs. A history of smoking was recorded if someone had smoked at any time in their lives. Diabetes mellitus was recorded in the case of a previous diagnosis of insulin-dependent or noninsulin-dependent diabetes, or repeated elevated levels of fasting glucose (>6 mmol/L). Presence of symptomatic coronary artery disease, peripheral arterial disease, and levels of cholesterol were recorded.

At the end of 2007 we had collected a total number of 149 lacunar stroke patients, which we expected to be sufficient for this observational study, because a previous study reported positive results on a population of 110 lacunar stroke patients.16

The study protocol was approved by the local ethical review committees of both hospitals. Informed consent was obtained from all participants.

Imaging

MR images were obtained and assessed by a previously described protocol17 a median of 30 days after the event (interquartile range, 8–76 days). Two experienced vascular neurologists assessed the MR images by consensus. The interobserver agreement, expressed by Cohen’s kappa (κ), was determined before this study: 0.89 for symptomatic infarct, 0.96 for the presence of ≥1 aLAC, 0.77 for periventricular WML, and 0.84 for deep WML.18

Subtyping Lacunar Stroke

Patients were categorized as extensive WML if the periventricular WML score of the modified Fazekas scale was 3 or the deep WML score of the Fazekas scale was 2 or 3. Patients were categorized as aLAC if ≥1 asymptomatic lacunar infarcts were present on MR imaging in the absence of WML. If neither aLAC nor extensive WML were present on MR imaging, then patients were categorized as ILA.

Samples

By venepuncture of a vein in the antecubital fossa, fasting blood samples were drawn in all patients at least 3 months after the ischemic event. Prescribed medication including antiplatelet agents and statins were continued at time of blood withdrawal. After discarding the first tube (to minimize puncture-related coagulation activation), the blood was divided over a tube containing citrate anticoagulant at low pH (to prevent formation of tPA–PAI-1 complex and preserving components of the fibrinolytic system; Stabilyte tube; Biopool) and a tube containing 3.2% sodium citrate. Platelet-poor plasma was prepared by a 2-step centrifugation process: 5 minutes at 2000 g and 10 minutes at 11 000 g at room temperature. Plasma aliquots were stored at −80°C and defrosted at 37°C before analysis.
Laboratory Assays
TF, TM, and PAI-1 antigen levels and tPA–PAI-1 complexes were measured by commercially available enzyme-linked immunosorbant assay kits (IMUBIND tissue factor enzyme-linked immunosorbant assay kit by American Diagnostic; Asserachom Thrombomodulin by Roche; TECHNOZYM PAI-1 antigen enzyme-linked immunosorbant assay reagent kit and tPA–PAI-1 complex enzyme-linked immunosorbant assay reagent kit, both by Technoclone). The tPA activity was determined with a chromolize tPA kit (Biopool). The vWF antigen was measured by a turbidimetric method and coagulation factor VIII by a 1-stage activated partial thromboplastin time (aPTT)-based clotting test, both on automatic analyzer (BCS analyzer; Siemens). The coefficients of variation for TF, TM, PAI-1, tPA–PAI-1 complexes, tPA activity, vWF, and coagulation factor VIII were <10%, <7.4%, 3% to 10%, 3% to 10%, <10%, <10%, 1.4% to 2.4%, and 2% to 14%, respectively.

Statistical Analysis
Normally distributed data are presented as mean±SD, variables with skewed distributions are presented as median and interquartile ranges, and categorical variables are presented as frequencies. We compared means by the independent t test for normally distributed variables and the Mann-Whitney test for variables with skewed distributions (tested by Kolmogorov-Smirnov-Test). Pearson χ² statistic was used for categorical variables. A 2-tailed P<.05 was considered significant.

For the error bars in the Figure, only normally distributed data could be used. Levels of vWF were normally distributed and levels of tPA and PAI-1 became normally distributed after logistic transformation.

We modeled the relation between the levels of endothelial markers for lacunar stroke subtypes (ILA vs extensive WML-positive and ILA vs aLAC). Statistical significance of these relationships was assessed using binary logarithmic regression analyses. The model was adjusted for age, gender, hypertension, diabetes, cholesterol levels at admission, and smoking history. Covariates were forced into the models simultaneously (enter method in SPSS). Analyses were performed using the SPSS statistical software package (version SPSS 16.0 for Windows; SPSS Inc).

Results
Clinical Characteristics
Fifty-three patients had extensive WML, 53 had ≥1 aLAC without WML, and 43 patients were classified as having ILA. In 26 (17%) patients, MR imaging showed no symptomatic lacunar infarct, and the diagnosis was based on clinical criteria. Patients with extensive WML were older than patients with ILA (68.2±9.3 for extensive WML and 58.9±9.9 for ILA; P<0.001). Distributions of gender, conventional vascular risk factors, and levels of cholesterol were similar between lacunar stroke subtypes (Table 1). Antiplatelet agents (100% of patients) and statins (90% of patients) were used at time of blood withdrawal. The fasting blood sample was taken 137 days (interquartile range, 106–206) after the stroke.

Levels of Plasma Markers of Endothelial Function and Coagulation Factor VIII
The tPA activity was elevated in patients with extensive WML (median, 0.79 IU/mL; interquartile range, 0.37–1.33) as compared to patients with ILA (0.44 IU/mL; interquartile range, 0.34–0.72; P=0.02) by Mann-Whitney test (Table 2, Figure). Differences in tPA activity were in the expected direction in patients with aLAC compared to ILA; however, they were not statistically significant (0.62 IU/mL; interquartile range, 0.31 to 1.06; P=0.21). After multivariable analysis (Table 3), the association between tPA activity and WML was no longer significant (OR, 2.82 per IU/mL; 95% CI, 0.96–8.25; P=0.06).

PAI-1 levels were lower in patients with extensive WML (27.5 ng/mL; 95% CI, 13.1–53.9) than in those with ILA (44.5 ng/mL; 95% CI, 22.9–84.3; P=0.02) by Mann-Whitney test. The association between WML and PAI-1 remained significant after multivariable analysis (OR, 0.99 per ng/mL; 95% CI, 0.98–1.00; P=0.04). Levels of PAI-1 in patients with aLAC were not significantly different from patients with ILA (41.4 ng/mL; 95% CI, 22.5–73.0; P=0.45).

Levels of vWF antigen were highest in patients with aLAC (148%±50 percentage of normal [%d.N]), followed by patients with WML (140%±51% d.N), and lowest in those with ILA (132%±52% d.N); however, differences were not sig-

### Table 1. Clinical Characteristics of Lacunar Stroke Subtypes

<table>
<thead>
<tr>
<th></th>
<th>Isolated Lacunar Infarct (n=43)</th>
<th>With Extensive WML (n=53)</th>
<th>P</th>
<th>With Asymptomatic Lacunar Infarcts, Without extensive WML (n=53)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>58.9±10.0</td>
<td>68.2±9.3</td>
<td>&lt;0.01</td>
<td>60.4±13.0</td>
<td>0.55</td>
</tr>
<tr>
<td>Male</td>
<td>26 (61)</td>
<td>30 (57)</td>
<td>0.70</td>
<td>37 (59)</td>
<td>0.34</td>
</tr>
<tr>
<td>Hypertension</td>
<td>27 (63)</td>
<td>36 (68)</td>
<td>0.60</td>
<td>35 (66)</td>
<td>0.74</td>
</tr>
<tr>
<td>Diabetes</td>
<td>6 (14)</td>
<td>6 (11)</td>
<td>0.70</td>
<td>7 (13)</td>
<td>0.92</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>5.6±1.1</td>
<td>5.8±1.2</td>
<td>0.50</td>
<td>5.7±1.4</td>
<td>0.80</td>
</tr>
<tr>
<td>Smoking</td>
<td>22 (54)</td>
<td>25 (48)</td>
<td>0.68</td>
<td>19 (36)</td>
<td>0.11</td>
</tr>
<tr>
<td>Coronary artery disease</td>
<td>4 (9)</td>
<td>8 (15)</td>
<td>0.39</td>
<td>7 (13)</td>
<td>0.55</td>
</tr>
<tr>
<td>Peripheral artery disease</td>
<td>1 (2)</td>
<td>2 (4)</td>
<td>0.69</td>
<td>2 (4)</td>
<td>0.69</td>
</tr>
</tbody>
</table>

Data are presented as mean±SD or N (%). P values for comparison with isolated lacunar infarct. WML indicates white matter lesions.
significant ($P=0.11$ for aLAC vs ILA and $P=0.41$ for WML vs ILA) by independent $t$ test.

Plasma levels of tPA–PAI-1 complex, TF, TM, and coagulation factor VIII were similar for subgroups of lacunar stroke patients.

**Discussion**

Several pathology\textsuperscript{7,19} and imaging\textsuperscript{20,21} studies provide evidence for the hypothesis that endothelial activation, eventually followed by leakage of plasma components into the vessel wall, perivascular space, and brain parenchyma, might be causative in the development of lacunar infarcts and WML.\textsuperscript{9} The function, and activation, of the vascular endothelium can be evaluated by functional tests such as flow-mediated vasodilatation or by measuring soluble markers secreted by the endothelium.\textsuperscript{10} In first-ever lacunar stroke patients, we found further evidence for this hypothesis because we found higher levels of tPA, a marker of endothelial function, in patients with concomitant extensive WML compared to patients with ILA.

To the best of our knowledge, we are the first to evaluate tPA as a marker of endothelial function in a substantial cohort of lacunar stroke patients. The elevated levels of tPA in patients with extensive WML coincided with low levels of PAI-1 in these patients. PAI-1 and tPA are extensively present in the small blood vessels of the white matter,\textsuperscript{22} and both proteins may be involved in mediating neuronal cell damage.\textsuperscript{23} Although the exact mechanisms are still unknown, low activity of PAI-1 is associated with tPA-induced tissue damage\textsuperscript{24} resulting from NMDA-induced ischemia by tPA after crossing the blood–brain barrier.\textsuperscript{25} Hence, we hypothesize that this mechanism might contribute to the development of WML through shifts in the balance between these components of the fibrinolytic system, ie, patients with WML lack the protective effect of PAI-1 for tPA-induced tissue damage.

Several studies\textsuperscript{26,27} found increased levels of vWF in lacunar stroke patient compared to controls. In our study, levels of vWF were higher in patients with extensive WML and aLAC than in those with ILA; however, differences were not statistically significant. The direction of the differences we found might suggest that endothelial activation is confined to the subtype with WML and/or aLAC.

Levels of soluble TF were similar for subtypes of lacunar stroke patients, which is in apparent contrast to those of Hassan et al\textsuperscript{16} who found higher levels of soluble TF in patients with increasing grades of leukoaraiosis. One confounding factor in this regard may be the use of medication. In our study, almost all patients used statins at time of blood withdrawal. Besides their lipid-lowering properties, statins reduce levels of TF,\textsuperscript{28} which may have contributed to the

<table>
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<th>Table 2. Levels of Plasma Markers of Endothelial Function and Coagulation Factor VIII in Lacunar Stroke Subtypes</th>
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<tr>
<td><strong>Isolated Lacunar Infarct</strong></td>
</tr>
<tr>
<td>(n=43)</td>
</tr>
<tr>
<td><strong>tPA activity, IU/mL</strong></td>
</tr>
<tr>
<td><strong>PAI-1, ng/mL</strong></td>
</tr>
<tr>
<td><strong>tPA–PAI-1-complex, ng/mL</strong></td>
</tr>
<tr>
<td><strong>vWF antigen, %d.N</strong></td>
</tr>
<tr>
<td><strong>FVIII, %d.N</strong></td>
</tr>
<tr>
<td><strong>TM, ng/mL</strong></td>
</tr>
<tr>
<td><strong>TF, pg/mL</strong></td>
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</table>

Data are presented as mean ($\pm$SD) or median (interquartile range).

$P$ value for comparison with ILA.

aLAC indicates asymptomatic lacunar infarcts; FVIII, coagulation factor VIII; PAI-1, plasminogen activator inhibitor type 1; TF, tissue factor; TM, thrombomodulin; tPA, tissue plasminogen activator; vWF, von Willebrand factor; %d.N, percentage of normal.

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<th>Table 3. Relationship Between Lacunar Stroke Subtype and Plasma Markers of Endothelial Function, Adjusted for Age, Gender, and Conventional Vascular Risk Factors</th>
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<tbody>
<tr>
<td><strong>Unadjusted Model OR (95% CI)</strong></td>
</tr>
<tr>
<td>Extensive WML</td>
</tr>
<tr>
<td>tPA</td>
</tr>
<tr>
<td>PAI-1</td>
</tr>
<tr>
<td>tPA–PAI-1-complex</td>
</tr>
<tr>
<td>vWF</td>
</tr>
<tr>
<td>TM</td>
</tr>
<tr>
<td>TF</td>
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</table>

Result of binary logistic regression analysis presented as OR with 1 unit of change in level of plasma marker (95% CI).

*P<0.05.
equal levels of TF in lacunar stroke subtypes. Similarly, we expected to find higher levels of soluble TM in patients with WML and/or aLAC compared to those with ILA, because several studies found elevated levels in lacunar stroke patients.16,26 However, although TM is expressed throughout the systemic circulation and microvasculature, its presence and distribution in the cerebral microvasculature is debated.29–31 Despite the fact that TM may be considered as an endothelial marker, it therefore might not be the most suitable marker for a disease of the cerebral small vessels.

The main strength of our study is that we were able to collect a substantial cohort of carefully subtyped lacunar stroke patients. Subtyping was performed by combining established criteria of the classical lacunar syndromes with imaging criteria and, second, by excluding patients with a possible embolic source of (lacunar) infarct, e.g., atrial fibrillation and carotid artery disease. This enabled us to define a strict phenotype of lacunar stroke patients who most probably had stroke because of an intrinsic disease of the cerebral small vessels. Our classification system was free of vascular risk factors, as suggested for studies on the pathophysiology of lacunar stroke.32 Furthermore, we assessed endothelial function after the acute phase and thereby can draw conclusions on the chronic process that might contribute to the development of the disease.

Our study has several limitations. First, we only selected patients with lacunar stroke and therefore could not compare or extrapolate our results to other types of ischemic stroke. Because aLAC and WML mainly appear in patients with lacunar stroke, and because cerebral small vessel disease underlies all, extrapolation to the other types of ischemic stroke is unwarranted. Second, our study is cross-sectional. As such, it cannot be established whether endothelial activation is causative of progressive. Long-term follow-up of patients with intermittent brain MR scanning might solve this problem. Third, the studied endothelial markers cannot be regarded as brain-specific endothelial cell markers. However, because several lines of evidence suggest that the small vessel disease that underlies lacunar stroke is a systemic disease,33–35 the pattern of endothelial cell activation also may be considered representative for the brain small vasculature. Fourth, despite the fact that we collected the largest series of well-subtyped lacunar stroke patients until now, we found differences in a limited number of endothelial markers. Because we based our sample size estimations on a study of 110 lacunar stroke patients,16 we expected that 149 patients would be sufficient. Pooling of our results with those from others may be a possibility to overcome this problem. Finally, 17% of patients had no symptomatic lacunar infarct on MR imaging. This may relate to the short duration of symptoms (at least 24 hours) in some, but also to the rather long MRI delay in some patients, which blurs the distinction between recent and possible concomitant old lacunar lesions. However, this did not lead to incorrectly included patients, because, clinically, they had experienced a lacunar stroke as diagnosed by established criteria.2

Conclusion

We found modest differences in levels of some of the circulating plasma markers of endothelial function in lacunar stroke patients with concomitant WML compared to ILA. Our findings seem to provide support for the hypothesis of endothelial activation in this subtype of lacunar stroke.

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Disclosure

None.

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