Markers of endothelial dysfunction in lacunar infarction and ischaemic leukoaraiosis

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Summary
Patients with cerebral small vessel disease (SVD) can present as isolated lacunar infarction or with diffuse white matter changes, with the imaging appearance of leukoaraiosis. Endothelial dysfunction, which can lead to breakdown of the blood–brain barrier, impaired cerebral autoregulation and prothrombotic changes, is believed to be important in mediating disease. Circulating levels of intercellular adhesion molecule 1 (ICAM1), thrombomodulin (TM), tissue factor (TF) and tissue factor pathway inhibitor (TFPI) are markers of endothelial activation and damage, and may provide insights into disease pathogenesis or differences between phenotypes. We therefore measured these markers in a prospective series of patients with lacunar stroke. One hundred and ten white Caucasian patients with previous lacunar stroke and 50 community control subjects were studied. Markers of endothelial function were measured on venous blood samples. Patients were classified on brain imaging into two groups: isolated lacunar infarction (n = 47) and ischaemic leukoaraiosis, defined as a clinical lacunar stroke and leukoaraiosis on brain imaging (n = 63). The number of lacunes and severity of leukoaraiosis were also scored on MRI. ICAM1, TM and TFPI were elevated in cerebral SVD subjects compared with controls (P < 0.006). The ischaemic leukoaraiosis group had a different endothelial marker profile, with lower levels of TFPI (P = 0.01) and a higher TF/TFPI ratio (P = 0.01) compared with the isolated lacunar infarction group. TM levels were associated with the number of lacunes (P = 0.008) and the leukoaraiosis score (P = 0.03), but TF levels and the TF/TFPI ratio were associated only with the extent of leukoaraiosis (P ≤ 0.02). These results suggest that there is evidence of chronic endothelial dysfunction in cerebral SVD, and endothelial prothrombotic changes may be important in mediating the ischaemic leukoaraiosis phenotype. Therapies which help to stabilize the endothelium may have a role in this group of patients.

Keywords: cerebral small vessel disease; lacunar infarction; ischaemic leukoaraiosis; endothelial dysfunction

Abbreviations: ECA = endothelial cell activation; ICAM1 = intercellular adhesion molecule 1; SVD = small vessel disease; TF = tissue factor; TFPI = tissue factor pathway inhibitor; TM = thrombomodulin

Introduction
Lacunar infarction, resulting from disease of the cerebral small vessels, accounts for a quarter of ischaemic strokes. It can occur in isolation, or be accompanied by changes in the periventricular white matter on CT or MRI. This appearance is called leukoaraiosis. Both lacunar stroke and leukoaraiosis are thought to be caused by cerebral small vessel disease (SVD). On pathology, thickening and hyaline deposition of the small perforating end arterioles supplying the white matter can be seen and, in some cases, localized small vessel microatheroma at the origin of the deep perforating arteries has been noted (Fisher, 1968, 1979) The neuropathological appearance corresponding to leukoaraiosis is neuronal loss, ischaemic demyelination and gliosis (Pantoni and Garcia, 1997). Both clinical and pathological studies support the hypothesis that lacunar infarction and ischaemic leukoaraiosis represent different forms of SVD.
Pathophysiologically, it is thought that a diffuse arteriopathy of the cerebral small vessels results in hypoperfusion and impaired autoregulation, and subsequent ischaemia (Pantoni and Garcia, 1997; Bakker et al., 1999; Terborg et al., 2000). If acute, this causes small focal regions of damage in perforating arteriole territories (lacunar infarction), while if it is more chronic it results in diffuse ischaemic injury (leukoaraiosis) (Brown, 1995). Leukoaraiosis itself is a radiological definition and can be caused by non-ischaemic pathologies. Therefore, the definition of ischaemic leukoaraiosis has been introduced to identify a group of patients in whom leukoaraiosis is likely to have an ischaemic basis. It is defined as radiological leukoaraiosis with a clinical lacunar syndrome (Jones et al., 1999).

The pathogenesis of cerebral SVD is poorly understood. Furthermore, it remains unclear why some patients develop isolated lacunar infarction while others also develop leukoaraiosis. Hypertension is the major risk factor for all types of cerebral SVD, but fails to account for much of the risk (Boiten et al., 1993; Boiten and Lodder, 1995). Novel risk factors including genetic predisposition (Carmelli et al., 1998) also appear to be important. A number of separate lines of evidence suggest that chronic endothelial dysfunction plays a pivotal role. The normal cerebral endothelium plays a crucial role in regulation of cerebral blood flow and autoregulation, and in the blood–brain barrier. In addition, in health, it presents an anticoagulant phenotype to blood. Under stimulation by numerous agents, the endothelium undergoes changes which allow it to participate in the inflammatory response; this is known as endothelial cell activation (ECA) (Hunt and Jurd, 1998). In cerebral SVD, there is histopathological evidence of ECA (Lin et al., 2000). One of the changes of ECA is increased vascular permeability, and it is thought that entry of serum proteins into the vascular wall and perivascular neural parenchyma may produce toxic effects (Tomimoto et al., 1996). Previous studies have shown that white matter cerebral blood flow (Markus et al., 2000) and autoregulation (Bakker et al., 1999; Terborg et al., 2000) are impaired in cerebral SVD, and endothelial-derived nitric oxide is believed to be an important mediator of both cerebral blood flow (White et al., 1998) and cerebral autoregulation in humans (White et al., 2000). Genetic studies involving humans (Elbaz et al., 2000) and knockout mice (Rudic and Sessa, 1999) also suggest that endothelial function may be important in mediating cerebral small vessel injury.

Endothelial activation and damage can be assessed in vivo by measuring soluble plasma markers which are released into the circulation (Cines et al., 1998). The different molecules reflect different aspects of endothelial dysfunction. Surface expression of intercellular adhesion molecule 1 (ICAM1) is a precondition for the adhesion and transendothelial migration of lymphocytes (Bevilaqua et al., 1989), and increased blood levels reflect an endothelial inflammatory response. Thrombomodulin (TM) is expressed normally on the endothelial cell surface where, with thrombin, it regulates the activity of protein C. Increased plasma levels are thought to reflect endothelial damage (Ishii et al., 1991). Tissue factor (TF) is not normally expressed by cells in contact with flowing blood but, under stimulation by agents such as tumour necrosis factor, TF can be expressed on monocytes and endothelium (Pearson, 1999). It acts as a trigger for the extrinsic coagulation pathway by binding to factor VII, and levels of soluble TF thus reflect prothrombotic change (Tremoli et al., 1999). The physiological inhibitor of TF is tissue factor pathway inhibitor (TFPI) which binds to activated factor Xa within the TF–VIIa–Xa complex, thus preventing thrombin formation (Bajaj et al., 2001).

The aims of this study were first to determine if there is evidence of endothelial dysfunction in patients with cerebral SVD, and secondly to determine if there are differences in the pattern of endothelial activation between patients with isolated lacunar stroke and patients with lacunar stroke and leukoaraiosis.

Methods

Study population

One hundred and ten white consecutive Caucasian patients with cerebral SVD attending out-patient stroke clinics at one of four participating hospitals were enrolled. Cerebral SVD was defined as a clinical lacunar syndrome (Bamford et al., 1987) with a compatible lesion on MRI/CT. All patients underwent full stroke investigation including brain imaging and imaging of the carotid arteries with duplex or magnetic resonance angiography. Exclusion criteria included subcortical infarction ≥1.5 cm diameter, cortical infarction of any size, a potential cardiac source of embolism as defined by the TOAST (Trial of Org 10172) classification (Adams et al., 1993) and large vessel cerebrovascular disease defined as carotid or vertebral artery stenosis >50%. Fifty white Caucasian community controls free of clinical cerebrovascular disease were also recruited by sampling of family doctor lists from the same geographical location as the patients. Sampling was stratified to a similar distribution of age, sex and conventional vascular risk factors as the patients in the study. Patients and controls with current clinical evidence of infection or a history of inflammatory or malignant diseases were not eligible for the study as this itself could result in ECA.

All patients and controls were reviewed by one physician and underwent a standardized clinical assessment. MRI was not performed in controls. Hypertension was defined as repeated measurement of systolic blood pressure >160 mmHg, diastolic pressure >95 mmHg (Subcommittee of WHO/ISH Mild Hypertension Liaison committee, 1993) or current treatment with antihypertensive drugs. A positive smoking history was recorded in those who had smoked at any time in their lives. Diabetes mellitus was defined as a previous diagnosis of insulin- or non-insulin-dependent diabetes. The study protocol was approved by local research...
ethics committees, and informed consent was obtained from all participants.

**Blood sampling and laboratory methods**

In all patients, blood was taken at least 2 months after the last clinical event (median 210 days) to avoid the transient early changes which have been observed in some endothelial markers in the first few weeks after acute stroke (Fassbender et al., 1995; Lindsberg et al., 1996). A 20 ml aliquot of blood was obtained and divided into polypropylene tubes for serum, and siliconized glass tubes containing 0.105 M sodium citrate for plasma collection. The specimens were centrifuged at 3000 g for 10 min and the isolated serum and plasma stored at −80°C until use. Levels of circulating markers were measured using ELISA (enzyme-linked immunosorbent assay)-based commercially available kits. ICAM1 measurements were performed on serum (R&D Systems), whilst TM, TF and TFPI levels were detected in plasma (Diagnostica Stago and American Diagnostica Inc.). The intra-assay (interassay) coefficients of variation for ICAM1, TM, TF and TFPI were <4.9% (<10.2%), <7.4% (<8.6%), <4.5% (<7.5%) and <7.2% (<7.4%), respectively.

**Assessment of CT and MRI scans**

MRI scans were available in 93 patients. The remaining 17 patients had CT alone. In all of the cases with MRI, axial T2-weighted images were evaluated blind to clinical and laboratory data by a single observer using a semi-quantitative rating scale. A modification of the Fazekas scale was used to score leukoaraiosis, as this scale has been shown to reflect pathological severity of cerebral SVD in a post-mortem validation study (Fazekas et al., 1993). Leukoaraiosis was rated as: 1 = absent or mild (equivalent to Fazekas periventricular score =2); 2 = moderate (Fazekas scale 3); and 3 = severe (more than half of the hemispheric white matter involved). In addition, we also scored the number of lacunar infarcts: 1 = ≤2 lesions; 2 = 3–5 lesions; 3 = ≥5 lesions. Separate scores were generated for small (<5 mm) and large (6–14 mm) lacunar infarcts. In a small number of patients (n = 17), MRI scans were not available and therefore periventricular low attenuation changes were rated on CT using the same scale.

**Subtyping of lacunar stroke**

To explore possible pathogenic differences between focal and diffuse disease, cerebral SVD patients were subtyped according to their scan appearances. Patients with isolated lacunar infarction were defined as those with a leukoaraiosis score of 1. Patients with ischaemic leukoaraiosis were defined as those having a leukoaraiosis score of 2–3, equating to a ‘moderate’ or ‘severe’ leukoaraiosis scale. Where CT only was available, subtyping was performed using these images. To ensure that this did not introduce an error in subtyping, a comparison of subtyping using CT and MRI was performed. Eleven patients were identified who had undergone CT within 3 months of their MRI scan. CT scans were assessed blind to clinical details, subtype allocation and MRI appearances. In all cases, patients would have been allocated to the same subtype (lacunar stroke or ischaemic leukoaraiosis) by either CT or MRI assessment. Twenty MRI scans were also selected randomly for re-evaluation by the same observer. There was perfect repeatability (κ = 1.0) for allocation of clinical phenotype. For the MRI disease scores, the repeatability (weighted kappa) was good for leukoaraiosis category (κ = 0.85), and moderate for large lacunar infarcts category (κ = 0.58) and small lacunar infarcts category (κ = 0.43).

**Statistical analysis**

The distributions of ICAM1, TM, TF and TFPI were positively skewed, therefore levels were log transformed to normalize distributions prior to analysis by parametric tests. When results were log transformed, they were expressed as geometric mean and 95% confidence intervals. The ratio of TF/TFPI was calculated to provide an index of coagulation activation. Chi square analysis was used to compare proportions, and the unpaired t test or ANOVA (analysis of variance) used to compare normally distributed data between two or more groups. Post hoc tests were performed using Scheffe’s method. Linear regression was used to determine association between mean marker levels and grade of disease. Relationships between markers were determined by calculating the Pearson correlation coefficient. Repeatability measures for phenotype assignment and MRI grading scales were based on kappa scores (Altman, 1991).

**Results**

**Subject characteristics**

The clinical characteristics of patients with cerebral SVD and of controls are shown in Table 1. There were no significant differences in age, gender or conventional cerebrovascular risk factors. Forty-seven cases were classified as isolated lacunar infarction, and 63 cases as ischaemic leukoaraiosis. The ischaemic leukoaraiosis group was significantly older than both controls and those with isolated lacunar infarction, but other risk factors were distributed similarly between the three groups. For those with MRI, the severity of leukoaraiosis was graded as 1 in 31 patients (33.3%), 2 in 47 patients (50.5%) and 3 in 15 patients (16.1%). For lacunar infarcts, 6–14 mm, the distribution of scores was grade 1, 66 patients (71%); grade 2, 20 patients (21.5%); and grade 3, seven patients (7.5%). For smaller lacunes (≤5 mm), the scores were 1, 11 patients (11.8%); 2, eight patients (8.6%); and 3, 74 patients (79.6%). There was no association between the degree of leukoaraiosis and either the large (P = 0.60) or small lacunar infarct grade (P = 0.81).
leukoaraiosis (P < 0.0005) compared with controls (Table 3). In contrast, TFPI levels were higher only in patients with lacunar infarction compared with either ischaemic leukoaraiosis (P = 0.01) or controls (P < 0.0005). The mean concentration of TF did not differ significantly between groups, but a higher TF/TFPI ratio was found in ischaemic leukoaraiosis compared with lacunar infarction (P = 0.01).

Mean TM levels were positively associated with both the leukoaraiosis and large lacunar infarct grades (P = 0.03 and P = 0.008), whereas TF levels and the TF/TFPI ratio were associated only with the extent of leukoaraiosis grade (P = 0.02 and P = 0.01) (Fig. 1). In a multivariate model which included age, sex, diabetes, hypertension, systolic and diastolic blood pressure, cholesterol, history of myocardial infarction, grade of leukoaraiosis and lacunar infarcts, the association between TM and lacunar infarct grade remained (P = 0.01), but it was weakened for leukoaraiosis (P = 0.07). Leukoaraiosis grade still remained associated with TF (P = 0.04) and the TF/TFPI ratio (P = 0.01). There was no association present between the small lacunar infarct score and any of the endothelial markers.

**Discussion**

Our findings demonstrate evidence both of systemic endothelial activation in patients with cerebral SVD, with
increased levels of ICAM1, TFPI and TM, and of different patterns of activation in patients with isolated lacunar stroke compared with those with additional leukoaraiosis. The increases in endothelial soluble markers were independent of conventional risk factors including hypertension and smoking. In small arterioles, the adherence of leukocytes and transendothelial migration are dependent on ICAM1 (Bevilacqua et al., 1989). Levels of soluble ICAM1 are believed to reflect changes in surface expression of the protein. Increased expression, which is part of ECA, would facilitate leukocyte-induced injury of small blood vessels and could lead to disruption of the blood–brain barrier. In this setting, high levels of TM are believed to reflect endothelial injury, whereas those of TFPI reflect endothelial stimulation or activation. Consistent with this, we found significant correlations between each of these endothelial markers.

The endothelial dysfunction we have demonstrated in patients with cerebral SVD is consistent with increasing evidence from a number of different approaches which suggests that endothelial activation is involved in the pathogenesis of cerebral SVD. In pathological studies, endothelial activation and damage with subsequent breakdown of the blood–brain barrier appear to be key features in cerebral SVD (Lin et al., 2000). It has been hypothesized that entry of macromolecules such as proteases, immunoglobulins, complement and cytokines into the vascular wall and perivascular neural parenchyma leads to impaired function and toxicity (Tomimoto et al., 1996). Genetic studies involving humans (Elbaz et al., 2000) and knockout mice (Rudic and Sessa, 1999) suggest that endothelial function is important in mediating cerebral small vessel type injury. Cerebral blood flow (Markus et al., 2000) and autoregulation (White et al., 1998) are both reduced in cerebral SVD; both are regulated by the endothelium, with endothelial release of nitric oxide playing an important role.

As well as examining the role of endothelial dysfunction in cerebral SVD, we examined whether there were differences in the pattern of endothelial dysfunction between patients with ‘isolated’ lacunar infarction and those with diffuse disease. The two clinical phenotypes represent different ends of the spectrum of cerebral SVD, and it has been suggested that different mechanisms may be important in the two conditions (Iwamoto et al., 1995; Tomimoto et al., 1999; Hassan et al., 2002). Patients with the ischaemic leukoaraiosis phenotype had lower TFPI levels and a higher TF/TFPI ratio than those with isolated lacunar infarction. One possible explanation could be that patients with ischaemic leukoaraiosis have less ECA than those with isolated lacunar infarction. However, as TFPI is an inhibitor of the TF–VIIa–Xa complex, a more likely explanation is that the ischaemic leukoaraiosis group have greater coagulation activation. Further evidence for this was provided when the radiological disease severity was examined in relation to the marker levels. The grade of leukoaraiosis was positively associated with TF levels and the TF/TFPI ratio. Recently, high levels of thrombin–antithrombin complex and prothrombin fragments

### Table 3

Mean concentration of endothelial markers in isolated lacunar infarction, ischemic leukoaraiosis and controls

<table>
<thead>
<tr>
<th>Marker</th>
<th>Controls ($n = 50$)</th>
<th>Isolated lacunar infarction ($n = 47$)</th>
<th>Ischemic LA ($n = 63$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICAM1 (ng/ml)</td>
<td>341.90 (320.63–364.50)</td>
<td>454.23 (417.54–494.31)</td>
<td>394.86 (362.49–452.06)</td>
</tr>
<tr>
<td>TM (ng/ml)</td>
<td>19.36 (16.37–22.91)</td>
<td>29.21 (25.02–34.11)</td>
<td>29.93 (25.96–34.58)</td>
</tr>
<tr>
<td>TFPI (ng/ml)</td>
<td>89.52 (84.47–94.86)</td>
<td>113.55 (102.54–125.72)</td>
<td>95.96 (88.82–103.68)</td>
</tr>
<tr>
<td>TF (pg/ml)</td>
<td>265.64 (214.98–328.25)</td>
<td>23.39 (189.01–286.20)</td>
<td>312.68 (256.74–380.72)</td>
</tr>
<tr>
<td>TF/TFPI</td>
<td>2.97 (2.39–3.68)</td>
<td>2.06 (1.65–2.56)</td>
<td>3.26 (2.61–4.06)</td>
</tr>
</tbody>
</table>

Levels expressed as geometric mean (95% CI). Overall $P$ (ANOVA) and within-group comparison (Scheffe’s post hoc test). LA = leukoaraiosis, LI = isolated lacunar infarction.
(1 + 2), which are markers of coagulation activation, were found in patients with ischaemic leukoaraiosis, compared with those with isolated lacunar infarction (Tomimoto et al., 1999). Local TF expression, as seen in disseminated intravascular coagulation, leads to local deposition of fibrin.

If this were to occur in ischaemic leukoaraiosis, then this would worsen the chronic hypoperfusion and ischaemia. Increased thrombin generation as a consequence of TF-mediated coagulation would also be expected to lead to enhanced platelet activation, and this feature has been reported in patients with diffuse cerebral SVD (Iwamoto et al., 1995). The source of the TF in ischaemic leukoaraiosis could be endothelial, as TF levels were associated with TM in this study. However, it is possible that TF is derived from other cell types such as monocytes.

Whilst disturbances of coagulation activation appeared to be specific for the ischaemic leukoaraiosis group, we found that increased markers of ECA and damage were common to both subtypes of cerebral SVD. No association was found between ICAM1 levels and the radiological extent of disease, perhaps reflecting the very high levels that were encountered in patients. However, TM levels were related to both the number of large lacunes (6–14 mm) and the radiological leukoaraiosis grade. As there is little within-subject variability in TM levels (Amiral et al., 1994), this molecule might be a useful candidate to monitor disease progression in patients.

In our study, we found no association between any of the markers and the grade of small lacunes (≤5 mm). One plausible explanation for this is that the underlying pathology of small lesions is highly heterogenous and may include dilated perivascular (Virchow Robin) spaces, gliosis and demyelination which are difficult to distinguish on T2-weighted images alone (Awad et al., 1986). However, focal hyperintensities >5 mm are likely to represent true lacunar infarcts associated with cerebral SVD (Braffman et al., 1988).

In this study, we used a carefully phenotyped population of patients and excluded individuals with any other possible causes of stroke. Blood sampling was performed in an outpatient setting, therefore a ‘healthier’ group of patients may have been over-represented. We chose controls from geographically similar communities, and included individuals with cardiovascular risk factors although individuals with prior cerebrovascular disease were excluded. Ideally, one would wish to control for asymptomatic cerebral SVD in these individuals by using brain imaging. However, the requirement for brain imaging could have reduced the recruitment rate and introduced bias in control recruitment. Therefore, we used a representative community control population. Even if some of our controls did have asymptomatic cerebral SVD, this would not alter our conclusions; in contrast, their inclusion might reduce the magnitude of the difference in endothelial markers between the two groups in our study.

In our population, many stroke patients were on antiplatelet or antihypertensive medications, which may have potential beneficial effects on endothelial function (Husain et al., 1998; Ruilope and Schiffrin, 2001). If this is correct, the true level of endothelial dysfunction in disease similarly may be even higher than that demonstrated in our study.

We studied patients at least 2 months after their last clinical event to ensure that any differences seen were not due to an acute phase response. Following experimental focal brain ischaemia (Okada et al., 1994) and human stroke (Fassbender et al., 1995; Lindsberg et al., 1996), there is increased expression of some of the endothelial markers that we studied, but this appears to be a transient phenomenon, which is completed within the first few days of ischaemic injury. We

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**Fig. 1** Association between marker levels and disease severity. *P = 0.03 LA, P = 0.008 LLI; **P = 0.02 LA; ***P = 0.01 LA. There were no other significant associations. LA = leukoaraiosis, LLI = large lacunar infarcts (6–14 mm). Grey = leukoaraiosis; white = lacunar infarcts (6–14 mm); black = lacunar infarcts (≤5 mm).
also found no relationship between marker levels and the time from stroke to blood sampling, consistent with the differences we found not being due to an acute phase response. Therefore, it is likely that the endothelial abnormalities demonstrated in this study are chronic.

A general limitation of using serum markers is that one cannot localize the source of the endothelial factors studied. Markers of endothelial dysfunction have been found to be elevated in large vessel coronary (Hwang et al., 1997) and cerebrovascular diseases (Hwang et al., 1997; Rohde et al., 1998; Fassbender et al., 1999a), although studies have been inconsistent (Rifai et al., 1999; Salomaa et al., 1999; Malik et al., 2001). It has also been suggested that sporadic SVD is a systemic disorder with involvement of peripheral small vessels, as is seen in CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy) (Ruchoux and Maurage, 1998). Therefore, the endothelial markers which we studied could have been derived as a consequence of vascular injury in any other organ bed and at any time, as well due to events in brain microcirculation. However, if endothelial dysfunction was involved in the pathogenesis of SVD, then treatments which improve endothelial function may offer novel therapeutic approaches. Soluble endothelial makers might also prove useful in monitoring disease progression, and as a surrogate marker in treatment studies.

The precise causes of endothelial dysfunction in our patient population need to be addressed in further studies. Marker levels were independent of conventional cerebrovascular risk factors. Newer risk factors such as homocysteine, which we did not control for, could be important. Very high levels of homocysteine were reported recently in patients with extensive SVD (Fassbender et al., 1999b), and homocysteine is believed to have toxic effects on endothelium in vivo and in vitro (Wall et al., 1980; Woo et al., 1997). Genetic factors may also be important. Several polymorphisms within genes involved in endothelial function have been reported as possible risk factors for lacunar stroke (Markus et al., 1995; Elbaz et al., 2000). Determining the precise causes of endothelial dysfunction in cerebral SVD is an important goal, as therapies which help to stabilize the endothelium may have a beneficial role in this patient population.

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