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Vascular inflammation in cerebral small vessel disease

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Abstract

Cerebral small vessel disease (CSVD) is considered to be caused by an increased permeability of the blood-brain barrier and results in enlargement of Virchow Robin spaces (VRs), white matter lesions, brain microbleeds, and lacunar infarcts. The increased permeability of the blood-brain barrier may relate to endothelial cell activation and activated monocytes/macrophages. Therefore, we hypothesized that plasma markers of endothelial activation (adhesion molecules) and monocyte/macrophage activation (neopterin) relate to CSVD manifestations. In 163 first-ever lacunar stroke patients and 183 essential hypertensive patients, we assessed CSVD manifestations on brain magnetic resonance imaging (MRI) and levels of C-reactive protein (CRP), neopterin, as well as circulating soluble adhesion molecules (sICAM-1, sVCAM-1, sE-selectin, sP-selectin). Neopterin, sICAM-1 and sVCAM-1 levels were higher in patients with extensive CSVD manifestations than in those without ($p < 0.01$). Neopterin levels independently related to higher numbers of enlarged Virchow Robin spaces ($p < 0.001$). An inflammatory process with activated monocytes/macrophages may play a role in the increased permeability of the blood brain barrier in patients with CSVD.

Keywords: Lacunar infarcts; White matter lesions; Adhesion molecules; Neopterin; Cerebral small vessel disease

1. Introduction

Cerebral small vessel disease (CSVD) poses major challenges for physicians in the aging population. CSVD has a high morbidity: it relates to (recurrent) ischemic stroke (de Jong et al., 2002), cognitive disturbance (Reed et al., 2004; Tullberg et al., 2004; Wen et al., 2004) and cognitive decline (Garde et al., 2005; van Dijk et al., 2008), gait disturbances (Baezner et al., 2008), and urinary problems (Poggesi et al., 2008). CSVD relates to vascular risk factors like increasing age (van Dijk et al., 2008; Wiszniewska et al., 2000) and hypertension (Boiten et al., 1993). However, effective therapies to reduce the burden of CSVD are lacking up until now, because the unraveling of the pathophysiological mechanisms leading to CSVD has only just started.

The pathogenesis of CSVD probably starts with an increase in permeability of the blood-brain barrier (Farrall and Wardlaw, 2009) with enlargement of Virchow Robin spaces (perivascular spaces), (a)symptomatic lacunar infarcts, white matter lesions (WML), and microbleeds as sequelae (Rouhl et al., 2009b; Vernooij et al., 2008; Wardlaw et al., 2003). The blood-brain barrier is maintained by the interplay between endothelial cells, pericytes, and astrocytes. Endothelial dysfunction could, therefore, lead to an increase in blood-brain barrier permeability. Indeed, in patients with WML, levels of soluble adhesion molecules,

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like sE-selectin (Fassbender et al., 1999), intercellular adhesion molecule-1 (sICAM-1) (Hassan et al., 2003), sP-selectin (de Leeuw et al., 2002), and vascular cellular adhesion molecule-1 (sVCAM-1) (de Leeuw et al., 2002), which are markers of endothelial dysfunction (Deanfield et al., 2007), are elevated. However, the cause of the endothelial dysfunction remains unclear.

Adhesion molecules are expressed by endothelial cells in increased amounts on activation. They enable interaction with circulating leukocytes (Ghosh et al., 1998). In patients with vascular diseases such as atherosclerosis and vasculitis, levels of circulating soluble adhesion molecules are elevated (Lind, 2003; Tervaert and Kallenberg, 1997). As a result of the leukocyte-endothelial interactions, both endothelial cells and leukocytes (e.g., monocytes) become increasingly activated (Schubert et al., 2010). Activated monocytes/macrophages produce neopterin as well as cytokines which induce liver cells to produce C-reactive protein (CRP) (Fuchs et al., 1992). Neopterin and CRP levels are elevated in patients with vascular disease, and relate to a higher risk of ischemic events (van Haelst et al., 2003). Furthermore, neopterin may itself induce endothelial dysfunction with increased expression of adhesion molecules as a consequence (Cirillo et al., 2006).

We hypothesized that patients with CSVD have higher levels of neopterin, CRP, and soluble adhesion molecules, than patients without. To test our hypothesis, we selected patients with a high prevalence of CSVD: first-ever lacunar stroke patients as well as essential hypertensive patients.

2. Methods

2.1. Patients

Patients included in our study participated in 2 larger studies, the lacunar stroke patients in a longitudinal study on biological determinants of CSVD, whereas the hypertensive patients participated in a study on brain damage in hypertension.

All 280 first-ever lacunar stroke patients (event between May 2003 and December 2007) who were registered in the prospective Maastricht Stroke Registry were eligible for inclusion in the study. This registry is a hospital-based database including all stroke patients over the age of 18 years with symptoms lasting longer than 24 hours. We defined lacunar stroke as an acute stroke syndrome with a lesion on imaging compatible with the occlusion of a single perforating small artery, consisting of a subcortical, demarcated lesion with a diameter $<$ 20 mm on magnetic resonance imaging (MRI). If no such lesion was visible, we used established clinical criteria for lacunar stroke (de Jong et al., 2002). Furthermore, causes other than CSVD (cardiac embolic source, cerebral large vessel disease, carotid stenosis on Duplex imaging; de Jong et al., 2002) or history of systemic vasculitis or malignant disease had to be excluded. Altogether, 163 patients were willing and able to participate.

Essential hypertensive patients were recruited from the outpatient department of Internal Medicine. At their inclusion, these patients were free of any symptomatic ischemic or vascular disease, or other comorbidity like atrial fibrillation, chronic renal disease, systemic vasculitis or malignant disease (Henskens et al., 2008). Of the 389 eligible patients, 183 were willing and able to participate in the present study.

Forty-three patients who visited the neurological outpatient department with myogenic back pain or entrapment neuropathies served as controls. They had no vascular or inflammatory disease, no hypertension, and no silent ischemic lesions on cerebral MRI.

Vascular risk factor profiles were recorded for all participants. For the lacunar stroke patients, we defined characteristics based on values obtained after the acute phase (at or around 3 months after the stroke). We defined hypertension as known hypertension, treated or not, or at least 2 blood pressure recordings $>$ 140/90 mm Hg; diabetes mellitus as known diabetes, treated or not, or fasting serum glucose $>$ 7 mmol/L, or a postprandial level $>$ 11 mmol/L on at least 2 separate occasions; coronary artery disease as known or treated angina pectoris, the presence of myocardial infarction, or typical electrocardiogram (ECG) changes; hypercholesterolemia as known high cholesterol levels, treated or known, or fasting total cholesterol levels of $>$ 5.0 mmol/L; and peripheral vascular disease as known intermittent claudication, leg ischemia at rest, or amputation as a consequence of peripheral vascular disease.

2.2. Procedures

To prevent confounding by acute phase responses, all lacunar stroke patients underwent the procedures mentioned below at or around 3 months after their stroke.

2.2.1. MRI of the brain

We used 1.5 Tesla imaging with both standard T2-weighted, fluid-attenuated inversion-recovery (FLAIR) and gradient echo sequences (Rouhl et al., 2009a). Images were assessed by consensus by 2 experienced neurovascular researchers (RPWR and RJvO) as described earlier (Rouhl et al., 2008); in case of disagreement, the judgment of a third (JL) was decisive. We counted silent lacunar infarcts (with diameter $<$ 20 mm; hyperintense lesions on T2 imaging with corresponding hypointense lesion with hyperintense rim on fluid-attenuated inversion-recovery images) and deep and/or superficial microbleeds (small, $<$5 mm hypointensities on gradient echo imaging, not representing calcifications or superficial blood vessels). We used the Fazekas scale to estimate the extent of the periventricular and deep WML (Fazekas et al., 1987). Extensive WML were defined as a score of 3 (periventricular hyperintensities with involvement of white matter) on the periventricular scale, and/or a score of 2 or 3 on the deep white matter scale (beginning confluence of lesions or large confluent lesions).

Furthermore, we determined another manifestation of cerebral small vessel disease (Rouhl et al., 2008), namely enlarged Virchow Robin spaces at 3 different levels (basal ganglia, linear spaces at sella media level, and punctuate spaces at centrum semiovale level). These were assessed for both hemispheres together using a predefined 3-point scale: (1) <20; (2) between 20 and 50; and (3) >50 (Rouhl et al., 2008).

2.2.2. Blood

Blood was sampled from an antecubital vein into 5-mL serum as well as 4-mL (ethylenediaminetetraacetic acid [EDTA]) plasma tubes (BD Biosciences, Breda, the Netherlands). Plasma was separated from the blood cells within 2 hours and stored at -70 °C whereas serum was stored at -20 °C until analysis.

2.2.3. Measurements of adhesion molecules: sVCAM-1, sICAM-1, sP-selectin, and sE-selectin

sVCAM-1, sICAM-1, sP-selectin, and sE-selectin were all measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits (BioSource, Europe, Nivelles, Belgium) following manufacturer's instructions. Results are expressed as ng/mL. Intra-assay variability was 3.1%, 4.1%, 2.4%, and 5.4%, whereas interassay variability was 5.2%, 7.7%, 5.2%, and 6.0% for sVCAM-1, sICAM-1, sP-selectin, and sE-selectin respectively.

2.2.4. Measurement of neopterin and high sensitivity (hs) CRP

Neopterin was measured using commercially-available enzyme-linked immunosorbent assay (ELISA) kits (IBL, Hamburg, Germany), following manufacturer's instructions. Data are expressed as ng/mL; for this test, intra-assay variability was 3.6%, whereas interassay variability was 7.2%. hsCRP was determined with nephelometry, using the BN ProSpec (Siemens, Erlangen, Germany) using a previously described protocol (Rothkrantz-Kos et al., 2003); data are expressed as mg/L. Intra-assay variability was 1.4%, and interassay variability was 0.9%.

2.3. Statistical analysis

First, in our primary unadjusted analysis, we compared levels of neopterin, hsCRP, and adhesion molecules (using Mann-Whitney tests) between all patients with or without (1) asymptomatic lacunar infarcts; (2) extensive WML; and (3) both asymptomatic lacunar infarcts in combination with extensive WML. In this analysis, we considered all lacunar stroke patients as well as all hypertensive patients together, which is in line with other studies in CSVD patients (like the Leukoaraiosis And DISability [LADIS] and Atherosclerosis Risk in Communities [ARIC] studies), assuming that the pathophysiology of CSVD manifestations is similar, regardless of the underlying disease (Gottesman et al., 2010; van Straaten et al., 2006). In a secondary unadjusted analysis, we considered the lacunar stroke patient group and hypertensive patient group separately. We used values from healthy controls as a reference for "normal" levels (without testing statistically, as we did not have a hypothesis regarding a difference between patients and controls). We also assessed unadjusted correlations (using Spearman's rho) between the adhesion molecules, hsCRP and neopterin.

Second, in our multivariate analysis we adjusted for possible confounders (age, sex, and vascular risk factor profile). In this analysis we tried to determine whether there were independent relations between adhesion molecules, neopterin or hsCRP on 1 hand and MRI characteristics of CSVD (asymptomatic lacunar infarcts, extensive white matter lesions, brain microbleeds and enlarged Virchow Robin spaces [VRs]) on the other, using linear regression analysis. We used log-transformation to correct for nonnormality of the data (neopterin and the adhesion molecules). Unless indicated otherwise, we considered a p value < 0.05 statistically significant. All analyses were performed using SPSS version 16 (Statistical Package for the Social Sciences; Chicago, IL, USA).

Table 1
Characteristics of lacunar stroke patients, hypertensive patients, and control subjects

	Lacunar stroke patients (n = 163)	Hypertension patients (n = 183)	Control subjects (n = 43)
Age, mean years (± SD)	63.9 (12.0)	55.3 (11.9)	62.0 (7.7)
Male sex	100 (61.0)	96 (52.5)	20 (45.5)
Hypertension	107 (65.2)	183 (100.0)	0 (0.0)
Coronary artery disease	23 (14.0)	19 (10.4)	0 (0.0)
Peripheral vascular disease	6 (3.7)	4 (2.2)	0 (0.0)
Diabetes	19 (11.6)	6 (3.3)	0 (0.0)
Hypercholesterolemia	129 (78.7)	72 (39.3)	10 (22.7)
Current smoking	62 (37.8)	35 (19.1)	13 (30.2)
Statin use	140 (85.4)	69 (37.7)	3 (6.8)
Antihypertensive use	110 (67.1)	163 (89.1)	0 (0.0)

Data are n (%) except where otherwise noted.

2.4. Ethical considerations

The study was approved by the Medical Ethical Committee of the Maastricht University Medical Centre. All patients and control subjects gave their written informed consent.

3. Results

3.1. Patient characteristics

Patient characteristics as well as vascular risk factor profiles are shown in Table 1. Of the lacunar stroke patients, 104 had asymptomatic lacunar infarcts, and 59 had extensive WML, whereas of the hypertensive patients, 13 had asymptomatic lacunar infarcts, and 22 had extensive WML. As per definition, none of the control subjects had asymptomatic lacunar infarcts or extensive WML.

Table 2
Median levels (with interquartile range within parentheses) of hsCRP, neopterin and adhesion molecules in different patient groups, classified by MRI

	Asymptomatic lacunar infarcts		Extensive WML		Both asymptomatic lacunar infarcts and WML		
	Yes (n = 117)	No (n = 229)	Yes (n = 81)	No (n = 265)	Yes (both) (n = 48)	No (none) (n = 196)	Healthy control subjects (n = 43)
hsCRP (mg/L)	2.13 (4.14)	1.90 (3.19)	1.95 (4.09)	1.94 (2.59)	2.21 (4.30)	1.92 (3.12)	2.70 (4.04)
Neopterin (ng/mL)	**2.01 (1.96)*****	**1.65 (0.62)**	**1.97 (1.01)*****	**1.71 (0.70)**	**2.05 (1.05)*****	**1.63 (0.62)**	1.70 (0.66)
sP-selectin (ng/mL)	**54.6 (62.1)***	**42.9 (46.9)**	45.3 (58.8)	47.3 (47.5)	45.6 (63.1)	42.8 (45.6)	62.0 (54.5)
sE-selectin (ng/mL)	24.2 (23.3)	25.0 (21.3)	28.4 (24.6)	24.1 (20.4)	28.9 (26.2)	24.6 (22.3)	28.8 (15.0)
sICAM-1 (ng/mL)	**548.7 (219.4)*****	**459.7 (192.0)**	517.3 (176.7)	473.7 (218.8)	**509.7 (171.7)****	**453.6 (187.8)**	524.3 (99.3)
sVCAM-1 (ng/mL)	**731.6 (274.5)****	**687.9 (212.3)**	**758.3 (305.7)*****	**692.4 (222.1)**	**742.5 (283.5)****	**681.6 (198.9)**	637.8 (147.1)

Data are median (interquartile range). Statistically significant differences are indicated in bold.
Key: CRP, C-reactive protein; hs, high sensitivity; WML, white matter lesions.
* $p < 0.05$ (yes vs. no).
** $p < 0.01$.
***$p < 0.001$.

3.2. Univariate associations between CSVD and adhesion molecules, neopterin, and hsCRP

In our first, unadjusted, analysis, we compared levels of inflammatory markers and adhesion molecules in patients with or without silent lesions on MRI. As shown in Table 2, patients with asymptomatic lacunar infarcts had higher levels of neopterin, sP-selectin, and sICAM-1 as well as sVCAM-1 than those without, whereas hsCRP and sE-selectin levels did not differ. When we compared patients with extensive WML with those without, the first had higher levels of neopterin and sVCAM-1. When patients with both asymptomatic lacunar infarcts as well as extensive WML were compared with those without both these types of lesions, neopterin, sICAM-1, and sVCAM-1 levels were higher in the first group. Neopterin as well as sVCAM-1 levels were similar in patients without CSVD manifestations as compared with healthy controls.

In a second unadjusted analysis, when considering lacunar stroke patients or hypertensive patients separately, only neopterin levels differed between those with or without asymptomatic lacunar infarcts only in patients with lacunar stroke ($p = 0.04$) and not in essentially hypertensive patients ($p = 0.10$).

3.3. Correlations between the different adhesion molecules, neopterin, and hsCRP

In general, all adhesion molecules, neopterin, and hsCRP correlated significantly with each other, though correlation coefficients are relatively low (Spearman rho ranges from 0.10 to 0.30). Only sVCAM-1 and neopterin had a rho of 0.30, with $p < 0.001$.

3.4. Multivariate analyses: neopterin and E-selectin independently relate to CSVD manifestations

We determined significant independent relations between the levels of adhesion molecules, inflammatory markers, and CSVD manifestations using linear regression analyses. The only association that remained significant after correction for age, sex, pre-existent large vessel disease (coronary artery disease or peripheral artery disease), as well as patient group (hypertensive or lacunar stroke patient), was that between higher neopterin levels and higher numbers of enlarged Virchow Robin spaces at the level of the basal ganglia ($\beta = 0.257$; $p < 0.001$), and that between higher sE-selectin levels and higher number of microbleeds, irrespective of their location (deep or superficial) ($\beta = 0.155$; $p = 0.045$).

4. Discussion

We found higher levels of neopterin in patients with asymptomatic lacunar infarcts as well as in patients with white matter lesions, than in those without these lesions. Furthermore, higher levels of sP-selectin, sE-selectin, and sICAM related to CSVD manifestations. However, most importantly, we found that after correction for vascular risk factors, neopterin levels related independently to higher numbers of enlarged Virchow Robin spaces at the levels of the basal ganglia. As neopterin is a marker of activated monocytes/macrophages our data suggest that activated monocytes/macrophages play a role in the pathophysiology of CSVD.

Limited data are available on neopterin in CSVD. In the acute phase of stroke, neopterin levels increase (Grau et al., 2001). However, there are no data on neopterin in the chronic phase after stroke or in relation to WML. We demonstrated that neopterin levels are higher in lacunar stroke patients than in hypertension patients. Furthermore, we found a relation between higher neopterin levels and higher numbers of enlarged Virchow Robin spaces at the basal ganglia level, which suggests a relation between monocyte/macrophage activation and CSVD. Neopterin is a product of activated monocytes/macrophages and it stimulates both the immune response as well as the endothelium (Hoffmann, 2007). Together with other products of acti-

vated monocytes/macrophages, neopterin may activate nuclear factor kappa-B, a transcription factor which induces endothelial activation with increased expression of adhesion molecules as a consequence (Cirillo et al., 2006). Endothelial activation in CSVD could result in increased permeability of the blood-brain barrier and induction of an inflammatory reaction, leading to dilation of the perivascular spaces. Enlargement of the Virchow Robin spaces coincides with inflammatory activity in multiple sclerosis (as evidenced by contrast enhancing lesions elsewhere in the brain) (Wuerfel et al., 2008). Thus, the independent relation between neopterin levels (activated monocytes/macrophages) and number of enlarged Virchow Robin spaces at the basal ganglia level suggests that there may be an inflammatory reaction in CSVD in which activated monocytes/macrophages play a role.

Strikingly, CRP values were not elevated in patients with CSVD manifestations, which contrasts with earlier observations (Schmidt et al., 2006). CRP is produced in response to interleukin-6 production by several types of cells among which are monocytes/macrophages. Neopterin, on the other hand, is produced solely by monocytes/macrophages as a result of stimulation by interferon-gamma, which, in turn, is produced by T-cells (Schroecksnadel et al., 2006). Because we observed a differential upregulation of inflammatory markers, activated T-cells could also be involved in CSVD. Importantly, in our study we avoided the pitfall of known causes for higher neopterin levels: increasing age, autoimmune disease (like antineutrophil cytoplasmic antibody [ANCA]-associated vasculitis; Muller Kobold et al., 1999), malignant disease, and infections (Fuchs et al., 1992). Except for these conditions, neopterin levels are fairly stable over time (van Haelst et al., 2003).

The inflammatory response we found could also relate to endothelial cell activation and dysfunction. Previously, others also demonstrated elevated levels of soluble adhesion molecules in patients with CSVD. In nondiseased subjects, sICAM-1 levels related to the extent of WML, independent of hypertension and age (Han et al., 2009). Furthermore, sICAM-1 levels are elevated after a lacunar stroke (Knottnerus et al., 2009), and higher sICAM-1 levels related to early neurological deterioration and worse outcome at 3 months (Castellanos et al., 2002). In lacunar stroke patients, sICAM-1 levels were higher in those with WML than in those without (Hassan et al., 2003). Moreover, in a community-based sample, higher sICAM-1 levels related to progression of WML and lacunar infarcts after 3 and 6 years (Markus et al., 2005). Thus, sICAM-1 seems to be an important disease marker in lacunar stroke and white matter lesions. However, the origin of sICAM-1 is heterogeneous: it may derive from endothelium, but also from activated monocyte/macrophages or from other cells such as smooth muscle cells (Constans and Conri, 2006). This may be the reason why earlier cross-sectional studies could not unequivocally demonstrate endothelial involvement in CSVD. We confirmed elevated sICAM-1 levels in patients with WML and lacunar infarcts, but unlike the aforementioned studies, we did not find an independent relation between sICAM-1 and manifestations of CSVD. This could be a result of our blood sampling protocol, as we avoided acute phase responses after stroke by postponing blood sampling and excluded patients with concomitant diseases. Also, we found an independent relation between sE-selectin and the number of microbleeds. sE-selectin provides the most valid indication of endothelial involvement, because its source is exclusively endothelial (Deanfield et al., 2007). Therefore, the independent relation between sE-selectin and the number of microbleeds strongly suggests activation of endothelial cells in CSVD.

Our study has several limitations. First, we confined stroke patient selection to those with lacunar stroke and hypertension, and therefore we are not able to generalize our findings to patients with other causes of stroke (atherothrombotic or cardioembolic) or white matter disease. Second, our study is cross-sectional, and therefore no firm conclusions can be drawn with regard to causality. Third, patient selection favored younger patients in the lacunar stroke group. However, this selection would rather lead to an underestimation of associations. Notwithstanding these limitations, the strengths of our study remain that we studied a large group of well characterized patients, and that we excluded effects of an acute phase response.

In conclusion, our study provides evidence for the involvement of activated monocytes/macrophages in cerebral small vessel disease. Though mechanisms have yet to be determined and our results need confirmation in other populations, this could be relevant for pathophysiological concepts of cerebral small vessel disease, and might lead to more effective therapeutic strategies to reduce the burden of CSVD in the aging population.

Disclosure statement

All authors report that they do not have conflicts of interest.

The study was approved by the Medical Ethical Committee of the Maastricht University Medical Centre. All patients and control subjects gave their written informed consent.

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