Angiogenic T-Cells and Putative Endothelial Progenitor Cells in Hypertension-Related Cerebral Small Vessel Disease

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Background and Purpose—Cerebral small vessel disease (CSVD) may be caused by endothelial dysfunction, whereas endothelial progenitor cells (EPC) may attenuate endothelial dysfunction. Their vitality is lower in CSVD. A subset of lymphocytes, angiogenic T-cells, is capable to stimulate EPC function. The purpose of our study was to explore the relation between CSVD manifestations, angiogenic T-cells, and EPC in hypertensive patients with CSVD.

Methods—We compared 32 essential hypertensive patients with CSVD (white matter lesions, asymptomatic lacunar infarcts, or microbleeds on 1.5-Tesla MRI) to 29 age-matched and sex-matched hypertensive controls. We counted angiogenic T-cells (CD3+/CD31+/CD184+) and putative EPC (CD31+/CD34+/CD45/KDR+) by flow cytometry and determined EPC vitality by in vitro cluster formation.

Results—Putative EPC numbers were lower in hypertensive individuals with CSVD than in those without (0.56±0.109/mL versus 13±6×10^3/mL [median±interquartile range]; P=0.011). Angiogenic T-cell numbers were also lower in hypertensive individuals with CSVD than in those without (0.56±0.25×10^3/mL versus 0.78±0.50×10^3/mL; P=0.008). Higher angiogenic T-cell numbers independently related to absence of CSVD (odds ratio, 0.088; 95% confidence interval, 0.012–0.627).

Conclusions—Our data suggest that angiogenic T-cells and putative EPC independently relate to radiological CSVD manifestations in hypertensive patients. (Stroke. 2012;43:00-00.)

Key Words: endothelial progenitor cells ▪ hypertension ▪ leukoaraiosis ▪ T-lymphocytes ▪ white matter lesions

Cerebral small vessel disease (CSVD) has several manifestations, such as white matter lesions, lacunar infarcts, and microbleeds. Blood-brain barrier dysfunction could play a key role in the pathogenesis of these abnormalities. Dysfunction of the blood–brain barrier may be attenuated by putative endothelial progenitor cells (EPC). Putative EPC are immature cells, which circulate in peripheral blood. They are involved in repair of endothelial damage and are possibly also involved in improving endothelial cell function. In CSVD, however, more severely affected patients have lower EPC vitality, whereas factors that regulate putative EPC in CSVD are largely unknown.

Recent studies suggest that angiogenic T-cells (T_ango) may regulate EPC function. T_ango express platelet endothelial cell adhesion molecule (CD31) as well as the receptor for stromal-derived factor 1 (CD184). Furthermore, T_ango promote the formation of new blood vessels and endothelial repair by stimulating the function of EPC.

We hypothesized that CSVD patients have lower numbers of T_ango, which may relate to lower EPC vitality. To test this hypothesis, we counted T_ango and putative EPC and measured EPC functional properties in 2 groups of hypertensive patients, 1 with and 1 without CSVD in a case-control design.

Subjects and Methods

We included 32 hypertensive patients with CSVD and 29 hypertensive patients without CSVD from a longitudinal cohort study on hypertensive brain damage. We defined CSVD as the presence of 1 or more of the following MRI characteristics: (asymptomatic) lacunar infarcts; extensive white matter lesions; and brain microbleeds. For putative EPC quantification, we used flow cytometry identifying putative EPC as CD31+/CD34+/KDR+. To determine the vitality of putative EPC, we used cell cultures with 2 different techniques and a telomerase assay to determine the degree of senescence in the cultured cells. To quantify T_ango we used flow cytometry, defining T_ango as CD31+/CD34+/CD184+.
Results

Patient Characteristics
Characteristics of the study population are provided online (Supplemental Table I). As a whole, risk factor profiles were similar between hypertensive patients with and without CSVD. Per definition, patients with CSVD had more extensive white matter lesions, more often had lacunar infarcts, and more often had microbleeds.

CSVD, EPC, and Circulating T<sub>ang</sub>
Data for T-cells and putative EPC in the patient groups with or without CSVD are shown in the Figure and the Table. Patients with CSVD had lower total T-cell counts, lower circulating T<sub>ang</sub> counts, as well as lower EPC counts. There was not a significant difference in ECFC or CFU-EC cluster counts or in EPC telomerase activity between the groups.

We performed binary logistic regression analyses to determine significant predictors for the presence of CSVD. After correction for total T-cell counts, as well as blood pressure measurements, only higher T<sub>ang</sub> numbers significantly related to the absence of CSVD (odds ratio, 0.088; 95% confidence interval, 0.012–0.627).

Discussion
In the present study, we observed that hypertensive patients with CSVD had lower T<sub>ang</sub> and putative EPC numbers in their blood than hypertensive control patients without CSVD, independently of blood pressure levels. Therefore, we postulate that T-cells, next to EPC, may be involved in the pathogenesis of hypertension related CSVD.

We investigated whether T<sub>ang</sub> might be a regulating factor of EPC number and functionality in patients with CSVD. These cells form a subset of T-cells, which might play a role in vascular repair, because they stimulate putative EPC in their restorative capacities as evidenced in an in vitro study. Angiogenic T-cell numbers decrease with age. We found that numbers of T<sub>ang</sub> were lower in hypertensive patients with CSVD, independent of age. Furthermore, recent studies suggest that T-cells are involved in the pathogenesis of hypertension and, in particular, in hypertension-associated

| Table. Cellular Variables for Patients With and Without Cerebral Small Vessel Disease |
|---------------------------------|---------|---------|---------|
| CSVD (n = 32)                  | No CSVD (n = 29) | P       |
| Circulating T-cells (10<sup>9</sup>/mL) median (IQR) | 1.14 (0.53) | 1.46 (0.54) | 0.026* |
| Circulating angiogenic T-cells (10<sup>9</sup>/mL) median (IQR) | 0.56 (0.25) | 0.78 (0.50) | 0.008* |
| Circulating endothelial progenitor cell concentration (10<sup>6</sup>/mL) median (IQR) | 0.010 (0.007) | 0.013 (0.006) | 0.011* |
| ECFC cluster counts (per well) median (IQR) | 64.5 (104.6) | 116.3 (88.0) | 0.129 |
| ECFC telomerase activity (standardized to 100) median (IQR) | 26.0 (38.0) | 34.0 (65.0) | 0.492 |
| CFU-EC cluster counts (per well) median (IQR) | 152.5 (81.3) | 122.3 (63.6) | 0.445 |
| CFU-EC telomerase activity (standardized to 100) median (IQR) | 52.5 (63.8) | 41.0 (58.0) | 0.527 |

*Significant differences. CSVD is defined as the presence (on MRI of the brain) of one or more of the following: white matter lesions, lacunar infarcts, or microbleeds.

CFU-EC indicates late adherent putative EPC in culture; ECFC, early adherent putative EPC in culture; CSVD, cerebral small vessel disease; IQR, interquartile range.
vascular damage. The relation between lower $T_{\text{ang}}$ and CSVD may specifically imply a role of $T_{\text{ang}}$ in CSVD. Still, the nature of this role remains elusive.

Our study has several limitations. First, our study design is cross-sectional and therefore the observed associations could also be an epiphenomenon of CSVD. Second, the definition of EPC remains controversial, hence the term putative EPC. However, for flow cytometry and for cultures we used established authoritative protocols. Third, although variables such as age, sex, and medication did not differ significantly between hypertensive patients with or without CSVD, we cannot exclude a mild effect of this difference because age and other risk factors inversely relate to EPC and $T_{\text{ang}}$ number. Furthermore, our study contains a relatively small number of patients, which could have led to statistical error. Therefore, future studies should be large enough to preclude such shortcoming. Notwithstanding these limitations, the strength of our study remains the novelty of our findings in a well-characterized group of hypertensive patients.

Conclusions
We found that T-cells and putative EPC relate to cerebral small vessel disease in hypertensive patients. In the process, $T_{\text{ang}}$ may play a role. These findings are novel and allow novel hypotheses regarding the pathophysiology of CSVD.

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Disclosures
None.

References
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SUPPLEMENTAL METHODS

Patients

Patients who were referred to the outpatient department of Internal Medicine for the evaluation of their elevated blood pressure were included between July 2004 and September 2006. Patients participated in a longitudinal cohort study on hypertensive organ damage in the brain. Patients with evidence of secondary hypertension, chronic renal insufficiency, diabetes, heart disease, atrial fibrillation or clinically evident cerebrovascular disease were excluded. Of the 389 eligible patients, 218 were willing and able to participate in the cohort study.

We selected two groups of patients at their follow-up visit, two years after their original inclusion. At this visit, patients underwent MRI of the brain as well as blood sampling. Fifty-three patients appeared to have CSVD (see below for criteria), and 32 of these participated in our study. In addition, age, sex and medication matched hypertensive control subjects without CSVD from the master study were asked to participate. Unfortunately, three subjects were unable to participate, which left a total of 29 hypertensive controls without CSVD. For the other 21 patients with CSVD there was no age, sex, and medication matched control available in the master study. With regard to their vascular risk factor profiles, these subjects (n=21) did not differ from the subjects with CSVD who were included (n=32).

As vascular risk factor profiles, we defined diabetes mellitus as known diabetes, treated or not, or fasting serum glucose >7 mmol/L, or a postprandial glucose level >11 mmol/L on at least 2 separate occasions; coronary artery disease as known or treated angina pectoris, myocardial infarction, or typical ECG changes; hypercholesterolemia as known high cholesterol levels, treated or not, 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.

Procedures

MRI of the brain

We described our MRI protocol in detail previously. In short, standard 1.5 Tesla T2-weighted, fluid-attenuated inversion-recovery (FLAIR), as well as gradient echo (T2*) sequences were used (Intera, Philips Medical Systems, Best, The Netherlands; scan parameters: 1.5 Tesla, field of view 23*23 cm, matrix 512*512, slice thickness 5 mm and gaps of 0.50 mm). Images were assessed by consensus by two experienced neurovascular researchers (RPWR and RJvO). We counted lacunar infarcts (with diameter < 20 mm; hyperintense lesions on T2 imaging with corresponding hypointense lesion with hyperintense rim on FLAIR images) and deep and/or superficial microbleeds (small (<5mm) 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. 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). Based on these assessments, we defined CSVD (and classified patients) with the presence of one or more of the following: (asymptomatic) lacunar infarcts, extensive white matter lesions or microbleeds.

Blood

For EPC quantification, 30 mL blood was sampled after an overnight fast, without the use of a tourniquet, and anticoagulated in heparin (standard 10 mL tubes, BD Biosciences, Breda,
The Netherlands). Serum samples were collected in 5 mL tubes (BD Biosciences). At the same time, we measured blood pressure by standard sphygmomanometry.

**Cell Culture: Measuring EPC cluster formation**

For cell culture, we used two different methods simultaneously. In short, peripheral blood mononuclear cells were isolated by density gradient centrifugation (Lymphoprep, Axis, Oslo, Norway) and plated onto gelatin (1%) coated wells at 4 x 10^6 cells per well (24 well plates) in 1 mL of medium (RPMI 1640 Glutamax I; Gibco/Invitrogen, Breda, The Netherlands) containing 20% heat-inactivated fetal calf serum (Integro BV, Lelystad, The Netherlands), 100 IU/mL penicillin and 100 µg/mL streptomycin (Gibco), heparin (20 IE/mL; Leo Pharma, Breda, The Netherlands) and endothelial growth factors (extracted from bovine hypothalamus). Cells were cultured at 37°C and 5%CO₂. After 48 hours, non-adherent cells were detached and replated onto new gelatin coated wells (at 1 x 10^6 cells/well). Adherent cells were supplied with fresh medium. Cultures were continued until day 7 for both the initially non-adherent cells (termed Endothelial Cell Colony-Forming Units (CFU-EC)); and the adherent cells (termed Endothelial Colony Forming Cells (ECFC)). All cultures were performed in duplicate. Clusters consisting of round cells, with emanating spindle shaped cells peripherally, were counted at 7 days, by 2 observers, blinded for all clinical data and each other’s countings. We expressed cluster counts as average of the duplex measurements counted by two different observers for further data analysis. The interobserver agreement (as measured by intraclass correlation) of this method was high: correlations were higher than 0.9 (p<0.001).

**EPC Senescence**

To assess senescence of the cultured cells, cells from the ECFC and CFU-EC cultures were harvested after 7 days of culturing. We used the commercially available telomerase assay TeloTAGGG telomerase PCR ELISAPLUS (Roche, Almere, The Netherlands). This assay detects telomerase activity with a lower activity denoting cellular senescence. The assay was performed according to the manufacturer’s instructions.

**Flow Cytometry: Counting EPC numbers**

To assess EPC numbers, we used the complete protocol previously published by Duda et al. In short, we separated cells from plasma, and applied Fc-blocking agent (Miltenyi Biotec, Bergisch Gladbach, Germany) before incubating with FITC-anti-CD31, PE-anti-KDR, PerCP-anti-CD34 and APC-anti-CD45 (all from BD Biosciences). While assessing EPC numbers, we adjusted for their low event rate in flow cytometry, by using IgG isotype controls for all fluorochromes (BD Biosciences). After washing, we counted cells with FACSCalibur (BD Biosciences) and gated mononuclear cells, identifying EPCs as CD31^+/CD34^{bright}/CD45^{low}/KDR^+. We corrected these cell numbers for aspecific binding by subtracting positive cells in isotype controls.
Figure 1: Gating strategy used for the flow cytometric enumeration of putative EPC (CD31+/CD34+/CD45-(or low)/KDR+). This analysis was performed by one trained observer, using the following standardized gating strategy. Panel A: gating of mononuclear cells (lymphocytes/monocytes) by their forward/sideward scatter properties. These cells are then gated (Panel B) for CD31+ and CD45- (or low). Sequentially, these cells are then gated for their KDR+ and CD34+ (Panel C). The marker settings for this last panel is determined by the isotype measurement (not shown) in which the population of cells is completely in the lower left quadrant. For putative EPC enumeration, the possible rare events in the upper right quadrant from the isotype measurement are subtracted from the events in the upper right quadrant of panel C.

**Counting Angiogenic T-cells**

We quantified angiogenic T cells (T_{ang}) by flow cytometry. We used the monoclonal antibodies PerCP-anti CD3, FITC-anti CD31, and PE-anti CD184 (all from BD Biosciences). We defined T_{ang} as CD3+CD31+CD184+ (strategy for gating shown in Figure 2). We quantified T_{ang} from peripheral blood as well as from EPC cultures. Cells were harvested from the plates after 7 days culturing.

Figure 2: Gating strategy used for the flow cytometric enumeration of T_{ang}. Panel A shows the gating of lymphocytes, based on their forward and side scatter properties. These cells are gated for positivity for CD3 (Panel B), the general T cell marker. Subsequently, these cells (R5 in panel B) are gated for CD31+/CD184+, upper right quadrant of panel C, which represents T_{ang}. 
**Statistical Analysis**
As data were not normally distributed, we evaluated whether significant differences existed between the cellular variables of patients with and without CSVD by means of the Mann-Whitney test. Subsequently, we determined independent predictors for the presence of CSVD using binary logistic regression analyses, with the presence or absence of CSVD as dependent variable and the cellular variables (numbers of T cells, \(T_{\text{ang}}\) and EPC) as covariates, with correction for blood pressure levels (both systolic and diastolic) and number of antihypertensive drugs, age and sex. All analyses were performed using SPSS version 16.0.

**Ethical Considerations**
The study was approved by the Medical Ethical Committee of the University Hospital Maastricht and Maastricht University. All patients and controls gave their written informed consent.
**SUPPLEMENTAL TABLES**

Table 1: Patient Characteristics

<table>
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<th>CSVD (n=32)</th>
<th>No CSVD (n=29)</th>
<th>p-value</th>
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<tr>
<td>Age (mean ±standard deviation (SD))</td>
<td>65.2 (9.3)</td>
<td>63.0 (7.6)</td>
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<td>Male sex (n (%))</td>
<td>18 (56.2)</td>
<td>15 (51.7)</td>
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<td>Coronary artery disease (n (%))</td>
<td>5 (15.6)</td>
<td>4 (13.8)</td>
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<td>Peripheral Vascular Disease (n (%))</td>
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<td>0 (0.0)</td>
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<td>Diabetes (n (%))</td>
<td>3 (9.4)</td>
<td>1 (3.4)</td>
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<td>Current Smoking (n (%))</td>
<td>5 (15.6)</td>
<td>2 (6.9)</td>
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<td>Systolic Blood Pressure (mmHg; (mean ±SD))</td>
<td>159.5 (17.7)</td>
<td>152.8 (17.3)</td>
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<td>Diastolic Blood Pressure (mmHg; (mean ±SD))</td>
<td>90.8 (10.9)</td>
<td>87.2 (9.8)</td>
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<td>Duration of hypertension (years; (mean ±SD))</td>
<td>12.4 (12.9)</td>
<td>14.0 (14.9)</td>
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<td>Duration of antihypertensive medication (months; (mean ±SD))</td>
<td>93.1 (113.8)</td>
<td>97.1 (125.3)</td>
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<td>Statin Use (n (%))</td>
<td>13 (40.6)</td>
<td>16 (55.2)</td>
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<td>ACE inhibitor Use (n (%))</td>
<td>9 (28.1)</td>
<td>9 (31.0)</td>
<td>1.000</td>
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<td>Angiotensin Receptor Blocker Use (n (%))</td>
<td>15 (46.9)</td>
<td>16 (55.2)</td>
<td>0.671</td>
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Table 2: Imaging Characteristics of the two patient groups. For the grading of WML, the Fazekas scale was used. Periventricular white matter: 0=no WML, 1=caps or pencil thin lining, 2= smooth halo, 3=extending into deep white matter. Deep white matter: 0=no WML, 1=punctuate lesions, 2=beginning confluence of lesions, 3= large confluent areas. *chi-square test.

<table>
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<th>p-value</th>
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<td>Asymptomatic Lacunar Infarcts (n (%))</td>
<td>6 (18.8)</td>
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<td>Periventricular White Matter Lesions (n (%))</td>
<td>0 (3.1)</td>
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<td></td>
<td>3 (12.5)</td>
<td>0 (0.0)</td>
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<tr>
<td>Deep White Matter Lesions (n (%))</td>
<td>0 (12.5)</td>
<td>17 (58.6)</td>
<td>&lt;0.001*</td>
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<td>1 (18.8)</td>
<td>12 (41.4)</td>
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<td>3 (12.5)</td>
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<td>Microbleeds (n (%))</td>
<td>11 (34.4)</td>
<td>0 (0.0)</td>
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REFERENCES


