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Increased Levels of Circulating Angiogenic Cells and Signaling Proteins in Older Adults with Cerebral Small Vessel Disease

THESIS

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ABSTRACT OF THE THESIS

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Background: Cerebral small vessel disease (SVD) is associated with increased risk of stroke and dementia. Progressive and insidious damage to the cerebral microvasculature may also trigger angiogenic processes to promote vessel repair. Elevated circulating endothelial progenitor cell (EPC) levels are observed in response to vascular injury and may represent a vascular protective mechanism. Pro-angiogenic signaling proteins may similarly be elevated with increasing damage to cerebral microvasculature. We aimed to quantify circulating EPCs and examine circulating levels of proangiogenic proteins in older adults with evidence of SVD.

Method: Independently-living older adults (ages 55-90) free of dementia or clinical stroke were recruited from the community and underwent venipuncture and brain MRI. Flow cytometry quantified circulating EPCs as the number of cells in the lymphocyte gate positively expressing EPC surface markers (CD34+CD133+CD309+). Plasma was assayed for proangiogenic factors (VEGF-A, VEGF-C, VEGF-D, Tie-2, Flt-1). Total SVD burden score

was determined based on MRI markers, including white matter hyperintensities, cerebral microbleeds and lacunes.

Results: Sixty-four older adults were included. Linear regression revealed that older adults with higher circulating EPC levels exhibited greater total SVD burden (β = 1.0 x 105, 95% CI [0.2, 1.9], p = .019), after accounting for age and sex. Similarly, a positive relationship between circulating VEGF-D and total SVD score was observed, controlling for age and sex (β = .001, 95% CI [0.000, 0.001], p = .048).

Conclusion: These findings suggest that elevated levels of circulating EPCs and VEGF-D correspond with greater cerebral SVD burden in older adults with no history of clinical stroke or dementia. Additional studies are warranted to determine whether activation of systemic angiogenic growth factors and EPCs represents an early attempt to rescue the vascular endothelium and repair damage in cerebral SVD.

INTRODUCTION

Cerebral small vessel disease (SVD) occurs commonly with advancing age and is associated with increased risk of cognitive impairment, vascular dementia and Alzheimer's disease, contributing to up to 45% of all cases of dementia[1,2]. Small vessel changes often remain asymptomatic, but are evident on brain magnetic resonance imaging (MRI)[3] and believed to result from pathological changes in the perforating cerebral arteries, penetrating arterioles, capillaries, and venules[4]. Microvascular function is crucial to addressing cerebral metabolic demands, clearing waste products and maintaining the blood-brain barrier[3,5–9]. Reduced blood flow and hypoxia due to degradation of small blood vessels may trigger angiogenesis—the process of blood vessel growth—as a compensatory mechanism[10]. While it is still debated whether the process of angiogenesis is beneficial or detrimental in the context of vascular brain injury, animal models and studies of large vessel disease show increased angiogenesis in response to vascular injury[11].

One feature of angiogenesis is the mobilization of cells that can repair the vasculature. Endothelial progenitor cells (EPCs) represent a heterogeneous cell population known to be mobilized in response to vascular-endothelial damage to promote vessel repair and regenesis[12]. Increased levels of circulating EPCs at day 7 post-intracerebral hemorrhage predict improved functional outcome at 12 months[13] and mobilization of EPCs has been observed in response to cerebral large vessel disease[14]. Similarly, animal mouse models of ischemic stroke suggest that transplantation of *in vitro* cultured EPCs attenuates blood-brain barrier leakage, tight junction protein degradation, and neurological deficits[15,16].

Similarly, damage to the cerebral vasculature can trigger the release of signaling proteins that are involved in angiogenesis. The vascular endothelial growth factor (VEGF) family proteins, as well as other angiogenic factors, are also known to induce endothelial and neuronal remodeling after stroke[17]. Levels of these proteins are elevated after ischemic insult in brain tissue and serum in rodent models and in humans[18–20]. However, few previous studies have evaluated whether changes in circulating angiogenic cells and proteins also occur in response to progressive and insidious cerebral SVD changes which commonly occurs with advancing age.

The current study evaluated whether circulating levels of EPCs and pro-angiogenic proteins are associated with SVD burden in older adults who were otherwise neurologically healthy, with no history of clinical stroke or dementia. Consistent with prior studies, we hypothesized that higher levels of circulating angiogenic proteins and EPCs would be associated with greater cerebral SVD burden.

METHODS

Participants

Participants were recruited from the community and included if they were 55 years of age or older, independently living with no history of clinical stroke, dementia or other systemic or neurological illness that may impact central nervous system function. History of vascular risk factors, including hypertension, body mass index, dyslipidemia, diabetes, as well as history of other medical illnesses, was determined by clinical interview. This study

was approved by the local Institutional Review Board; all participants gave informed consent and underwent blood draw and brain magnetic resonance imaging (MRI).

Endothelial Progenitor Cell Quantification

Venipuncture was performed after an overnight fast. EPCs were quantified with flow cytometry. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation and washed twice with DPBS + 2% FBS at 120 x g for 10 min and 300 x g for 8 min at room temperature. Fluorescently labelled antibodies to EPC surface antigens were utilized[21]. 1 million cells were transferred to an unstained tube blank, and the remainder was transferred to the stained tube, where 5 µL of Human BD Fc Block (BD Biosciences) was added and incubated for 10–15 min. An additional 1 µL of each of the following antibodies were then added: (1) CD34-PE-Vio770 (clone: AC136, Miltenyi Biotec), (2) CD133-VioBright FITC (clone: AC133, Miltenyi Biotec), (3) PerCP/Cy5.5-CD309 (clone: 7D4-6, BioLegend). Tubes were incubated in the dark for 30 min at 4 °C. Samples were washed with 3 mL PBS, centrifuged at 300 x g for 8 min, and fixed with 2% formaldehyde in PBS until analysis. Compensation controls were conducted using AbC Total Antibody Compensation Bead Kit (ThermoFisher). Samples were acquired on a BD LSR II flow cytometer and analyzed on FlowJo software. EPCs were defined as CD34+CD133+CD309+ cells[12]. Circulating EPCs were quantified by flow cytometry as the number of cells in the lymphocyte gate positively expressing EPC surface markers (CD34+CD133+CD309+). CD34+CD133+CD309+ cell concentrations were calculated as a percentage of lymphocytes.

APOE Genotyping

APOE genotyping was conducted on the blood cell pellet fraction obtained from plasma separation. DNA was isolated from the pellet fraction using the PureLink Genomic DNA Mini Kit (Thermo Fisher). Genotyping was conducted on isolated DNA using the TaqMan SNP Genotyping Assay (Thermo Fisher) on an Applied Biosystems 7300 Real Time PCR System. APOE gene SNPs were assessed for dbSNP IDs rs429358 and rs7412. Allelic discrimination was conducted using the included qPCR software. The APOE-ε4 allele was designated as rs429358-C + rs7412-C.

Angiogenic Protein Levels

Levels of angiogenic proteins in plasma were determined using the Meso Scale Discovery V-PLEX Human Biomarker 40-Plex Kit Angiogenesis Panel 1 (VEGF-A, VEFG-C, VEGF-D, Tie-2, Flt-1, PIGF, bFGF), following manufacturer's protocol without modification. Briefly, we utilized the MSD Multi-Spot 96-well 7-spot plate pre-coated with capture antibodies. Plates were washed 3 times with at least 150µl/well of wash buffer. 50µl of diluted plasma samples per well were added for the angiogenesis panel and incubated at room temperature with shaking for 2 hours. Plates were then washed 3 times with at least 150µl/well of washing buffer. 25µl of detection antibody solution was added to each well and incubated at room temperature with shaking for 2 hours. Plates were then washed 3 times again. 150µl of 2X Read Buffer T was added to each well. Plates were then analyzed on an MSD instrument. Values below the lower limit of detection (LLOD) were replaced with zero.

Cerebral Small Vessel Disease Quantification

All participants underwent a comprehensive neuroimaging protocol as previously described[21]. The following sequences were examined for the current analysis: (1) 3D T1-weighted anatomical scan for qualitative assessment of brain structures and abnormalities, (2) T2-weighted scan for identification and differentiation of lacunes, (3) fluid-attenuated inversion recovery (T2-FLAIR) for the evaluation of white matter hyperintensities, and (4) T2*-weighted imaging for assessment of cerebral microbleeds. MRI markers were identified and scored in accordance with established neuroimaging standards for SVD[22]. To determine total MRI SVD burden, all imaging markers were combined using a SVD score (amended version) recently developed by Olama et al.[23], which ranges from 0 to 7 and includes grading of white matter hyperintensities (0-3; using the Fazekas scale), number of lacunes (0-3; 0 = no lacunes, 1 = 1-2, 2 = 3-5, 3 = > 5) and presence of microbleeds (0-1; 0 = absent, 1 = present).

Statistical Analyses

All analyses were performed using IBM SPSS Statistics 27 and R Version 3.6.1. Data were initially screened for extreme values (outliers) defined as values greater than 5 standard deviations from the mean and values that were unduly affecting regression parameter estimates based on measures of distance or influence. Two participants had extreme values for CD34+CD133+CD309+ cell counts, two participants had extreme values for VEGF-C and one participant had an extreme value for VEGF-D and were excluded from analyses. We examined the relationship between circulating EPCs as well as angiogenic proteins (independent predictors) and SVD score (dependent outcome), independently and after adjusting for age and sex. In addition, we examined any significant effects of APOE4

carrier status on the relationship between angiogenic circulating cells as well as proteins and SVD burden. Significance threshold was set at $p < 0.05$ for all analyses.

RESULTS

A total of 64 participants were included in the analysis. Age of study participants ranged from 55 to 90 years (Mean (M) = 69.8, standard deviation (SD) = 7.3), education ranged from 6 to 20 years (M = 15.8, SD = 2.8 and 40.6% were male (Table 1).

Cerebral Small Vessel Disease Burden

SVD markers were evident (SVD score > 1) in 29 (45.3%) participants (Figure I). SVD scores ranged from 0 to 4 (M = 1.6, SD = 1.0). Microbleeds were identified in 5 (7.8%) participants, and small lacunes in 8 (12.5%) participants. White matter hyperintensities were identified in majority of participants; 36 (56.3%) displayed mild white matter hyperintensity burden (Fazekas 1), 18 (28.1%) displayed moderate burden (Fazekas 2) and 6 (9.4%) showed severe burden (Fazekas 3).

Association Between EPCs and Small Vessel Disease Burden

EPC count (CD34+CD133+CD309+ count per lymphocyte) and SVD score was available for 50 participants after outlier removal. Mean EPC count was 2.7×10^{-6} (SD = 3.4×10^{-6}). Simple linear regression revealed that higher levels of circulating EPCs was associated with greater SVD burden, even after accounting for age and sex in multiple regression ($\beta = 1.0 \times 10^{5}$, 95% CI [0.2, 1.9], $p = .019$). The relationship remained significant even after adjusting for APOE4 carrier status and a significant positive effect of APOE4

carrier status was observed, with APOE4 carriers having greater SVD burden (N = 48; β = 0.57 , 95% CI [0.01, 1.13], *p* = .019; Table II).

Association Between Pro-Angiogenic Proteins and Small Vessel Disease Burden

We specifically examined the relationship of VEGF-A, VEFG-C, VEGF-D, Tie-2 and Flt-1 with SVD score. Values were available for all 64 participants, however two participants had extreme values for VEGF-C and one participant had an extreme value for VEGF-D and were excluded from the current analyses. Simple linear regression revealed a positive relationship only between circulating VEGF-D and total SVD score, which remained significant in multiple regression controlling for age and sex (β = .001, 95% CI [0.000, 0.001], *p* = .048). The relationship remained significant even after adjusting for APOE4 carrier status (Table III).

DISCUSSION

Mobilization of EPCs and release of angiogenic growth factors has been observed in response to cerebral large vessel disease[14]. Consistent with such studies, our findings indicate increased levels of circulating EPCs and angiogenic proteins in the presence of cerebral SVD. Specifically, circulating numbers of CD34+CD133+CD309+ cells and levels of VEGF-D protein correlated with greater cerebral SVD burden based on white matter hyperintensities, cerebral microbleeds and lacunar infarction. SVD markers were evident on MRI in almost half our sample, yet participants did not have major cognitive dysfunction at the time of assessment, suggesting that the correlation between cerebral SVD and these angiogenic factors may be observed during the preclinical disease stage. While the current

study was correlational, precluding causal inference, our findings are consistent with the hypothesis that EPCs and angiogenic signaling proteins may be increased in circulation in response to cerebral SVD[21].

Studies focused on clinically symptomatic patients with more severe white matter changes, cognitive impairment and dementia indicate a limited pool of EPCs may be exhausted with the emergence and progression of clinical symptomatology[24]. Based on these data and the present study findings, we hypothesize that increased circulating EPC and VEGF-D levels in asymptomatic older adults may represent preclinical markers of early-stage cerebral small vessel injury, and that later exhaustion of the EPC pool may coincide with progressive cognitive decline. The continued elevation of VEGF-D in the presence of cognitive impairment is supported by prior research, although the literature suggests that EPC levels deplete in correspondence with the decline in cognitive function. If our hypothesized model is supported, the potential for circulating EPC and VEGF-D levels biomarkers for early-stage cerebral SVD changes warrants further research. Moreover, animal studies suggest that transplantation of EPCs may attenuate blood brain barrier breakdown[15], tight junction protein degradation, and neurological deficits[15,16], suggesting that EPCs may also offer potential therapeutic opportunities, but additional studies are needed.

Increased levels of VEGF-D have been associated with atrial fibrillation, ischemic stroke and heart failure[25,26] and elevated levels are known to predict mortality in patients with coronary artery disease[27]. VEGF-D is a secreted glycoprotein and one of five members of the VEGF family with high angiogenic and lymphangiogenic potential[28]. Recent animal

and in vitro studies also suggest a role for VEGF-D in dendrite maintenance required for memory formation and other cognitive processes[29–31]. It remains unclear whether circulating EPCs may play related roles in central nervous system functions. In the present study, we demonstrate an association between circulating VEGF-D and SVD in older adults, suggesting that angiogenic and lymphangiogenic processes may be taking places. However, further studies are warranted to confirm these findings and uncover potential mechanisms.

Whether the mobilization of EPCs and angiogenic factors to promote angiogenesis is beneficial or detrimental in SVD warrants further attention. Angiogenic processes, including endothelial barrier function and cell connections that may be weakened during repair and remodeling, may lead to inflammation and increased blood-brain barrier permeability[11]. The involvement and impact of angiogenic cells and signaling proteins in repairing cerebral SVD—often a progressive form of vascular damage which worsens with age—may be important in understanding cognitive decline in older adults.

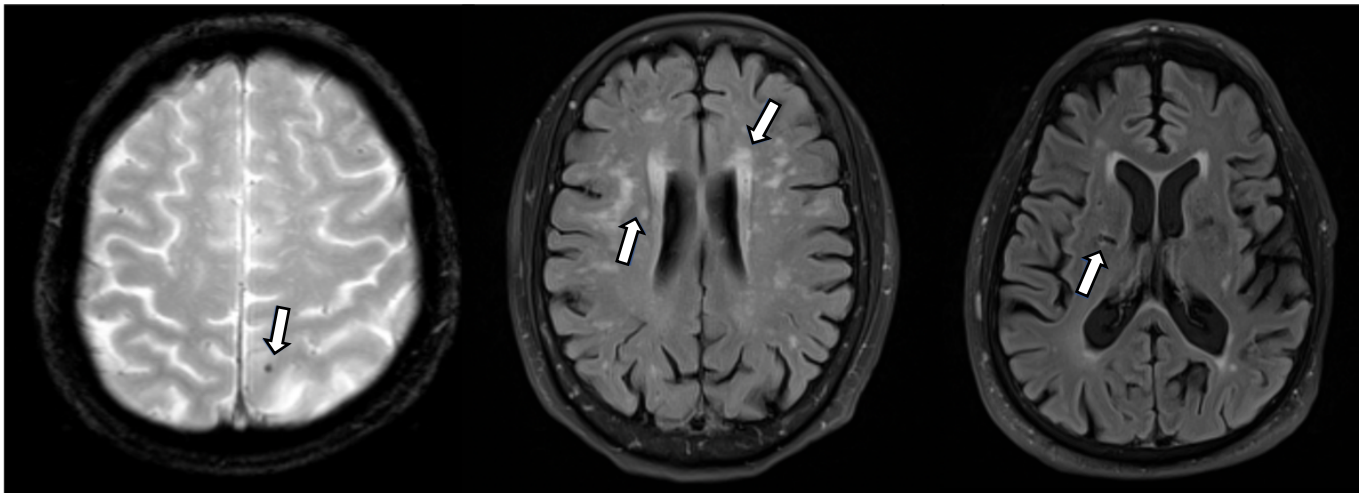
Limitations of this study include the limited sample size and cross-sectional design. Further studies may elucidate the predictive value of these angiogenic biomarkers. The findings of this study provide further insight into the potential role of angiogenesis in microvascular pathology of the aging brain.

Table I. Participant Characteristics, Demographics and Vascular Risk Factors

	N = 64
Age, M (SD)	69.8 (7.3)
Sex Male, n (%)	26 (40.6)
Education, M (SD)	15.8 (2.8)
Hypertension, n (%)	26 (40.6)
Dyslipidemia, n (%)	33 (51.6)
Diabetes, n (%)	6 (9.4)
Smoking History, n (%)	26 (40.6)
TIA, n (%)	1 (1.6)
Cardiovascular Disease, n (%)	5 (7.9)
Atrial Fibrillation, n (%)	3 (4.8)
Left Ventricular Hypertrophy, n (%)	1 (1.6)

[1]History of cardiovascular disease, atrial fibrillation and left ventricular hypertrophy was collected for 63 (98.4%) participants and missing for 1 participant.

Figure I. Greater Small Vessel Disease as Identified on MRI



Note. A*. microbleed identified on T2*;* ***B****. white matter hyperintensities identified on FLAIR;* ***C****. lacune on FLAIR*

Table II. Association Between EPCs and Small Vessel Disease

Variable	Unstandardized Coefficients		Standardized B	Sig.	95% Confidence Interval for B	
	B	Std. Error			Lower Bound	Upper Bound
Age (years)	.004	.019	.033	.838	-.035	.043
Sex (male)	-.153	.269	-.080	.572	-.696	.389
APOE4 (carrier)	.568	.276	.291	.046	.011	1.125
Circulating EPCs (CD34+CD133+CD309+/lymphocyte)	105447.5	41519.0	.394	.015	21716.5	189178.4

Note. *Dependent Variable: SVD Score*

Table III. Association Between VEGF-D and Small Vessel Disease

Variable	Unstandardized Coefficients		Standardized B	Sig.	95% Confidence Interval for B	
	B	Std. Error			Lower Bound	Upper Bound
Age (years)	.031	.018	.236	.092	-.005	.067
Sex (male)	-.078	.255	-.039	.761	-.589	.434
APOE4 (carrier)	.330	.283	.159	.249	-.237	.898
Circulating VEGF-D (pg/mL)	.001	.000	.257	.048	.000	.001

Note. *Dependent Variable: SVD Score*

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