Age-dependent expression of VEGFR2 in deep brain arteries in small vessel disease, CADASIL and healthy brains

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Abstract

Vascular myocytes are central to brain aging. Small vessel disease (SVD; arteriolosclerosis) is a widespread cause of lacunar stroke and vascular dementia, and is characterised by fibrosis and depletion of vascular myocytes in small penetrating arteries. Vascular endothelial growth factor (VEGF) is associated with brain aging, and VEGFR2 is a potent determinant of cell fate. Here, we tested whether VEGFR2 in vascular myocytes is associated with older age and SVD in human brain.

VEGFR2 immunolabelling in deep grey matter was assessed in older people with or without moderate-severe SVD, or in younger people without brain pathology or with a monogenic form of SVD (CADASIL). All cases were without Alzheimer's disease pathology. Myocyte VEGFR2 was associated with increasing age (p=0.0026) but not with SVD pathology or with sclerotic index or blood vessel density. We conclude that VEGFR2 is consistently expressed in small artery myocytes of older people, and may mediate effects of VEGF on brain vascular aging.

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Keywords: small vessel disease; vascular dementia; arteriolosclerosis; vasculopathy; Flk-1

1. Introduction

Cerebral small vessel disease (SVD; arteriolosclerosis) is a widespread ageing-related vasculopathy that causes lacunar stroke [13, 25], diffuse white matter lesions [31, 35] and vascular cognitive impairment and dementia (VCID) [11, 17]. SVD is characterised by fibrotic thickening in the wall of small penetrating arteries, with depletion of vascular smooth muscle cells (VSMC) [18, 25, 26]. Though increasing age and high blood pressure are risk factors for SVD, the molecular drivers for these cellular changes are unknown.

The trans-membrane tyrosine kinase-linked receptor VEGFR2 (also known as KDR or Flk-1) is a potent determinant of cell fate [30, 41]. Activation of VEGFR2 in endothelial cells by the canonical ligand VEGF is associated with angiogenesis and increased vascular permeability. Within CNS tissues VEGFR2 has been reported in neurones [4, 6, 24, 27, 39], oligodendrocyte progenitors [19], microglial cells [12] and in VSMC within spinal cord [6] and retinal arteries [8]. The ligand VEGF is also expressed in brain tissue [3, 23, 38].

In adult brain VEGF is considered to play a role in neurorepair, following cerebrovascular injury [15]. Following focal ischemia in cerebral cortex of non-human primates, VEGF and VEGFR2 are both augmented [38, 39], and VEGF is elevated in cerebrospinal fluid (CSF) of ischemic stroke patients[9]. A role for VEGF in age-related dementia also appears likely[23]. In a recent longitudinal study of older people, VEGF concentration in CSF was positively associated with better brain aging outcomes, including episodic memory and executive function [21].

Here we observed VEGFR2 in vascular smooth muscle cells (VSMC) within small penetrating arteries in caudate-putamen of older people, a subcortical grey matter region known to develop SVD [13, 25]. We hypothesized that VEGFR2 may influence cell fate in the aging brain vasculature, and we therefore tested whether VEGFR2 in VSMC is associated with age and with SVD.

2. Methods

2.1 Human brain tissue

Post mortem brain tissue was obtained from: The Oxford Brain Collection, John Radcliffe Hospital; and from Newcastle Brain Tissue Resource Centre, Newcastle upon Tyne. Brains from older people (age at death: 65 y or more) were derived from the OPTIMA cohort [20]. Older people with neuropathological diagnosis of moderate-severe SVD were of average age (± SD): 80.1 ± 10.7 y (range 66 – 99 y, n=15, 10 male:5 female). Older people without neuropathological SVD: 79.5 ± 5.8 y (68 – 89 y, n=12, 6 male:6 female). Younger people without brain pathology: 30.0 ± 12.3 y (male aged 11 y (M11), F26, M32, M40, F41). CADASIL patients: 56.5 ± 9.9 y (F44, M55, M59, F68). This is a well-preserved tissue cohort that we have examined previously [5, 14]. All tissue samples were donated following written informed consent by donors or their next of kin. This study had approval of Local Research Ethics Committees and the UK National Research Ethics Service.

We examined paraffin wax-embedded tissue blocks containing anterior caudate-putamen. All brains were fixed in 10% neutral buffered formalin for at least 1 month, prior to wax embedding. Mean post mortem delay was 42 ± 23 h (mean, SD; range 18 – 115 h) and mean formalin time from post mortem examination to wax embedding was 6 ± 2 months (range: 1 – 9 mo). All cases were free from Alzheimer's neuropathology as defined by CERAD guidelines and NIA-Reagan designation. Three of the older SVD cases were neuropathologically graded as Braak stage III/IV for Alzheimer Disease (AD)-related neurofibrillary tangle pathology. All other cases were Braak stage ≤ II. In all cases, cerebral amyloid angiopathy was reported as "absent/mild". CADASIL cases had typical SVD pathology and there was no evidence of any other lesions, including AD type neuropathology [42]. In all cases endothelia were positive for CD34 immunolabelling (not shown), suggesting intact antigenicity.

2.2 Neuropathological assessment of SVD

SVD was defined on microscopic examination of standard haematoxylin and eosin sections by a registered Neuropathologist (Dr C Joachim or MME), using a semi-quantitative assessment scale [11]. SVD was defined as: widened perivascular spaces, or hyaline thickening of arteriolar walls plus mild-moderate perivascular pallor of myelin staining, or loosening with attenuation of nerve fibres with gliosis in white matter or loss of nerve cells and gliosis in deep grey matter in one or more sections. Absence of SVD was defined as: no widening of perivascular spaces or hyaline thickening of arteriolar walls, no perivascular

pallor of myelin staining, loosening of tissue or attenuation of nerve fibres, or gliosis in white matter, or loss of nerve cells and gliosis in deep grey matter [5, 11, 14].

2.3 Antibodies

VEGFR2 primary antibody (rabbit monoclonal; clone 55B11) was from Cell Signalling Technology, Danvers, MA. The immunogen was a GST-fusion protein containing the C-terminal 150 amino acids of human VEGFR2. This antibody detected a double band at the appropriate molecular weight corresponding to VEGFR2 protein in Western blots, with minimal background labelling, and did not cross-react with VEGF-R1 or VEGF-R3 [32]. CD34 antibody (clone QBend10, mouse monoclonal IgG_1) was from Novocastra-Leica Microsystems, Newcastle upon Tyne, UK. GFAP (rabbit polyclonal, Z0334) was from Dako-Cytomation, Glostrup, Denmark and smooth muscle α-actin (SMA; mouse monoclonal, clone 1A4) was from Sigma-Aldrich, Poole, UK.

2.4 Immunohistochemistry

Paraffin wax embedded sections (6 µm) were de-waxed and processed for standard immunohistochemical staining [5, 14]. After exposure to H_2O_2 (0.5 mol/L) to abolish endogenous peroxidise activity and high-pressure heat-induced antigen retrieval (0.5 min, 120 ^{o}C, pH 7.8) sections were blocked with bovine serum albumen (BSA; 3 % w/v) in phosphate-buffered saline containing 0.1 % v/v Triton-X100 (PBT). VEGFR2 primary antibody was diluted (1:600) in PBT containing 3 % w/v BSA and applied to sections overnight (4 ^{o}C). Other primary antibodies were applied for 60 min at room temperature: CD34 (1:100), GFAP (1:10,000), smooth muscle α-actin (1:500). Antibody labelling was visualised using a peroxidise-conjugated secondary reagent and diaminobenzidine (DAB) chromogen (Envision® kit, K4065, Dako, Carpinteria, CA), then counterstained with Mayer's haematoxylin. As a negative control neighbouring sections were treated without any primary antibody or with irrelevant primary antibody (rabbit anti-sheep IgG, 1:100; BD-Pharmingen).

2.5 Morphometric methods

DAB-labelled sections were examined on a Zeiss Axioplan-2 microscope driven by Axiovision software (version 4.7). Within each VEGFR2-labelled section, all vessels of arterial appearance with outer diameter between 20 and 200 µm, with a clear, non-inflected

cross-sectional profile, were photographed in a blinded fashion. We term these vessels "small arteries", assumed to encompass arterioles. To assess the abundance of VEGFR2 in myocytes, two blinded observers (FAJ; NSJ) independently scored the degree of immunoreactivity as absent, sparse, moderate or abundant. VEGFR2 scoring was absolute (not relative), in that no attempt was made to correct for reduced numbers of myocytes in a given vessel. Of the 513 vessels graded, the two observers differed by at most one category (on 65 vessels, 12.7 %), and for all these consensus was reached by discussion.

Sclerotic index (defined as 1 - inner diameter/outer diameter) is a measure of SVD severity at the single-vessel level [14, 17, 26, 28]. Sclerotic index was determined for all vessels of arterial appearance, cut with a clear circular profile, with outer diameter in the range 20-200 µm [17]. Sclerotic index < 0.3 corresponds to normal vessels without SVD; 0.3-0.5 to moderate SVD; and >0.5 severe SVD [26, 28, 40].

2.6 Statistical analysis
Student unpaired t tests were used to test for significant differences (p<0.05) in continuous variables (sclerotic index, age, post mortem interval) between groups. Mann-Whitney and Kruskal-Wallis tests with Dunn's post hoc corrections were used for categorical variables (grades of VEGFR2 labelling).

3. Results

3.1 Histology and Immunohistochemistry
We examined caudate-putaminal tissue from 36 individuals. Older people (aged 65 y or more) were categorised in terms of the presence or absence of neuropathological moderate-severe SVD [11] referred to as SVD cases and aged controls (AC), respectively. Young control cases (YC) were without SVD or other brain disease. CADASIL cases were genetically confirmed for *NOTCH3* mutations, as detailed previously [42].

In all tissue samples examined some capillaries were positive for VEGFR2 labelling (within endothelial cells, examples in Figure 1A) though this was not universal, or widespread. This labelling pattern was absent if primary antibody was omitted or an irrelevant primary antibody was used (rabbit anti-sheep IgG, not shown). Within parenchymal tissue of older

people (AC and SVD groups) some astrocytes were positive for VEGFR2 (Figure 1B). VEGFR2 labelling in neurones was sparse (Figure 1A, B).

Cells in the medial layer of small vessels, with the appearance of VSMC, were frequently positive for VEGFR2. These medial VEGFR2 positive cells were seen in small vessels of older people, in CADASIL cases and in YC cases (Figure 1). Confocal imaging of tissue sections double-labelled for VEGFR2 and the smooth muscle marker α-actin (SMA) confirmed co-labelling in VSMC of small arteries (Supplementary Figure S1).

3.2 Morphometric analysis

Degree of VEGFR2 labelling of VSMC in small arteries was graded on a categorical scale as absent, sparse, moderate or abundant (examples in Figure S2). Across the four groups of cases (YC, CADASIL, AC and SVD) there was a significant difference in degree of VEGFR2 labelling (p=0.017; Figure 2A). There was no significant difference between AC and SVD groups (p=0.463). Aged cases (pooling data from AC and SVD groups) had higher median VEGFR2 score than younger cases (pooling YC and CADASIL groups) (Figure 2A; p=0.0026, Mann-Whitney test).

Blood vessel density was assayed using densitometry software (see Supplementary file) in neighbouring sections labelled with the endothelial marker CD34 and the VSMC marker SMA. There was no significant difference in extent of CD34 or SMA labelling with respect to degree of VEGFR2 labelling (p=0.432, 0.552 respectively).

Sclerotic index was higher in the SVD group relative to AC cases (p=0.045) and higher in CADASIL than in YC cases (p=0.023, Figure 2B). Pooling data from all aged cases (AC, SVD) sclerotic index did not differ in relation to VEGFR2 labelling grades (p=0.378, Figure 2C).

4. Discussion

Within adult CNS tissues the receptor VEGFR2 is seen in neurones of human, rat and primate brain [4, 24, 27, 39, 41], in oligodendroglial progenitors [19], in human microglia

[12] and in human spinal neurones [6]. VEGFR2 has also been observed within VSMC, in small arteries of human spinal cord [6] and in blood vessels of peripheral tissues [8, 36, 37]. The ligand VEGF is also expressed in brain tissue [3, 23, 38]. Within adult brain VEGF was detected in neurones and astrocytes [2, 4, 23, 27, 38].

The half-life of VEGFR2 protein is brief (a few hours) in cell culture systems [29, 36]. VEGFR2 transcriptional expression appears to be tightly controlled by multiple factors, including the hypoxia-inducible factor HIF2a and VEGF itself. Following agonist (VEGF$_A$) binding and receptor activation, VEGFR2 is rapidly internalised from the plasmalemma and degraded via a ubiquitin-dependent proteosomal pathway [10, 29]. Within native tissues the rate of VEGFR2 protein turnover may be slower, dependent on local binding partners and the degree of receptor activation [10, 29, 36].

The functional role of VEGFR2 within adult brain is unlikely to be angiogenic, as new vessel growth is sparse in adult nervous tissue. This is supported by our finding that endothelial VEGFR2 was not widespread. Physiological effects of VEGFR2 in adult brain include migration of neuronal progenitor and oligodendrocyte progenitor cells [19, 41].

Following injury or in disease states, several functional roles for central VEGFR2 are reported. Within multiple sclerosis lesions, endothelial VEGFR2 is induced by the inflammatory cytokine IL-1β, and drives local blood-brain barrier permeability (due to endothelial claudin-5 breakdown) [1, 2]. VEGFR2 expression is strongly induced in VSMC following IL-6 exposure [43], further supporting a role for VEGFR2 in the response to neuroinflammatory stimuli [30, 41]. VEGFR2 is also upregulated following acute ischemic challenge [15, 33, 39]. In cerebral cortical grey matter of non-human primates approximately 10 % of neurones were positive for VEGFR2, and this was enhanced following focal ischaemia, both within the ischemic lesion and in distant neurones functionally connected to the lesion site[39]. In the present study neuronal VEGFR2 labelling was rare, possibly reflecting differences in species, chronological age and the brain regions examined (here, a deep subcortical nucleus, the caudate-putamen).

Our finding of robust VEGFR2 expression in VSMC, a cell-type that is central to brain vascular aging, invites speculation. Our data do not support association with blood vessel density, or small vessel disease. Within experimental systems, the potent tyrosine kinase-

linked VEGFR2 has functional effects on migration, differentiation state and functional phenotype of VSMC [7, 8, 22, 36, 37, 43]. In human aortic VSMC cultures, and in adult mouse retina, VEGFR2 had a potent anti-migratory effect, mediated by VEGFR2-PDGFRβ receptor complexes [8]. In transgenic mice (VEGF$^{\delta/\delta}$) lacking physiological levels of VEGF expression, arterial VSMC lacked mature contractile phenotype, and their sympathetic neuro-effector junctions were abnormal in structure and function [37]. VEGF$^{\delta/\delta}$ animals had greatly impaired cerebral blood flow autoregulation, in response to a hypoxic challenge (4-fold lower than wildtype mice) [37]. These data suggest that in adult brain arteries an adequate level of VEGF is required to maintain VSMC in a stable, non-migratory state, and in a fully-differentiated, contractile phenotype [8, 37].

We speculate that the VSMC-expressed VEGFR2 we observe may be under tonic activation from centrally-produced VEGF. Previous work from cell culture and animal studies suggests that this may be required for normal neurovascular coupling, and to maintain the stable non-migratory, contractile phenotype of mature myocytes in penetrating small arteries [8, 37]. Myocyte VEGFR2 may also participate in VEGF-mediated actions in neurorepair, following cerebrovascular injury [15, 33]. Brain protective actions of VEGF are suggested by findings in mice [34] and human subjects [16, 21]. In a North American population of older people (the Alzheimer's Disease Neuroimaging Initiative, or ADNI, cohort) VEGF concentration in CSF was positively associated with better brain aging-related outcomes (hippocampal atrophy, and composite measures of episodic memory and executive function)[21]. We speculate that VEGFR2 in vascular myocytes may mediate some aspects of this protective action of VEGF.

In conclusion, myocytes in small arteries were strongly positive for VEGFR2 in deep grey matter of older people. The degree of expression was associated with age, and may be a factor in vascular changes relevant to the aging brain.

Acknowledgements

The authors have no conflict of interest.

Figure Legends

Figure 1. VEGFR2 immunohistochemical labelling in human caudate-putamen.

A, VEGFR2 (brown) is seen in capillary endothelial cells. B, VEGFR2 is evident in astrocytic cell bodies (arrows). In A and B large neuronal somata (N) are unlabelled. C, the wall of a small artery (Ar) but not that of a neighbouring small vein (Ve) are strongly positive for VEGFR2. D, in a small artery from a CADASIL case with severe fibrotic thickening in the vessel wall (arrow shows acellular fibrous material), myocytes are strongly positive for VEGFR2. E-H, VEGFR2 labelling in a small artery wall (panel E). This overlaps with smooth muscle actin (SMA) in a neighbouring section (F), but not with perivascular astrocyte labelling with GFAP (G). For orientation, a haematoxylin-eosin (HE) labelled neighbouring section is shown. A-G, haematoxylin nuclear counterstain. A, E-H: 68 year old male, moderate SVD. B: female, aged 90 y, moderate SVD. D: male CADASIL patient, age 55 y. Scale bars: 20 micron.

Figure 2. VEGFR2 labelling in relation to subject group, neuropathological SVD severity and sclerotic index.

A, Median grade for myocyte VEGFR2 immunohistochemical labelling, compared across four subject groups: young controls (YC), CADASIL cases, aged controls (AC) and aged individuals with moderate-severe sporadic small vessel disease (SVD). Each symbol represents a single subject. Horizontal lines mark the group median. Pooled older cases (AC, SVD) had significantly higher median VEGFR2 grade than pooled younger cases (YC, CADASIL). B, sclerotic index as a measure of single vessel wall thickening was compared across patient groups. Horizontal lines mark the group mean. Mean sclerotic index was higher in CADASIL than in YC cases, and higher in SVD than AC cases. Data from 511 vessels in total. C, sclerotic index did not differ across the different grades of myocyte VEGFR2 labelling. Data shown only for aged cases (AC, SVD). Box-whiskers show median, inter-quartile range and full range for each group. *$p < 0.05$, ** $p < 0.01$.
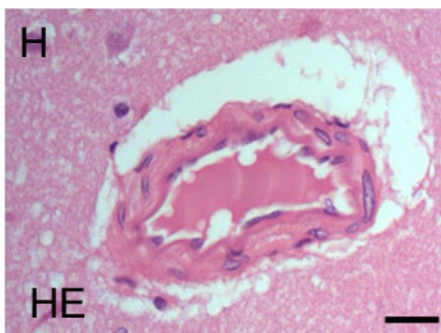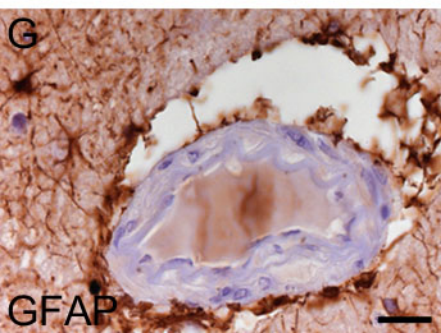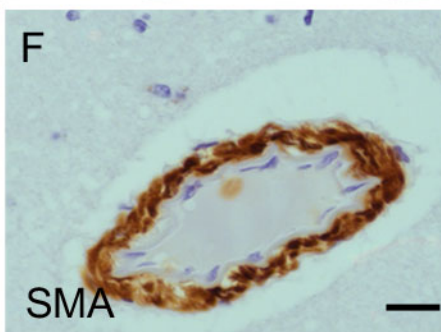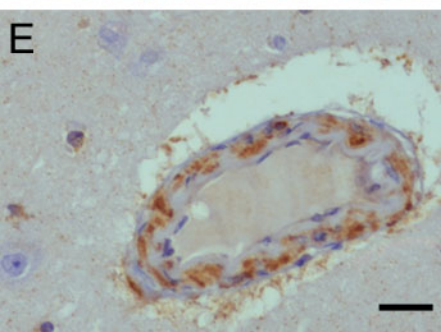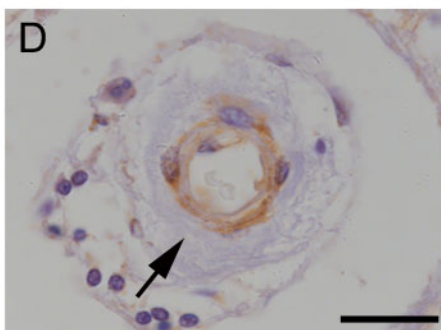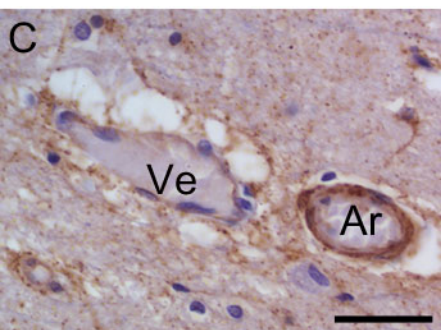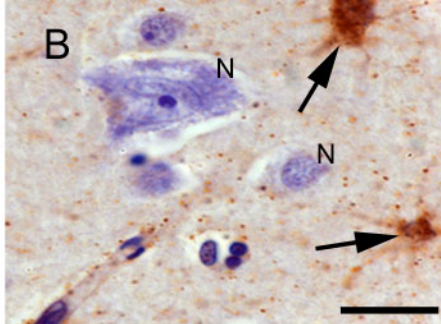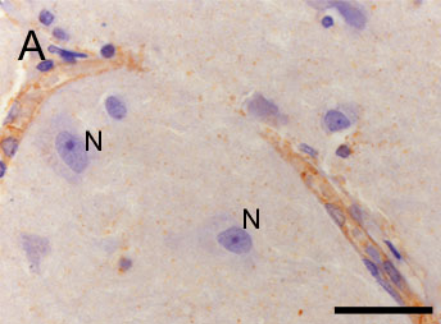
References

[1] Argaw AT, Gurfein BT, Zhang Y, Zameer A, John GR. VEGF-mediated disruption of endothelial CLN-5 promotes blood-brain barrier breakdown. Proc Natl Acad Sci U S A 2009;106(6):1977-82.

[2] Argaw AT, Zhang Y, Snyder BJ, Zhao ML, Kopp N, Lee SC, Raine CS, Brosnan CF, John GR. IL-1beta regulates blood-brain barrier permeability via reactivation of the hypoxia-angiogenesis program. J Immunol 2006;177(8):5574-84.

[3] Barker R, Ashby EL, Wellington D, Barrow VM, Palmer JC, Kehoe PG, Esiri MM, Love S. Pathophysiology of white matter perfusion in Alzheimer's disease and vascular dementia. Brain 2014;137(Pt 5):1524-32.

[4] Boer K, Troost D, Spliet WG, van Rijen PC, Gorter JA, Aronica E. Cellular distribution of vascular endothelial growth factor A (VEGFA) and B (VEGFB) and VEGF receptors 1 and 2 in focal cortical dysplasia type IIB. Acta Neuropathol 2008;115(6):683-96.

[5] Bridges LR, Andoh J, Lawrence AJ, Khoong CH, Poon WW, Esiri MM, Markus HS, Hainsworth AH. Blood-brain barrier dysfunction and cerebral small vessel disease (arteriolosclerosis) in brains of older people. J Neuropathol Exp Neurol 2014;73(11):1026-33.

[6] Brockington A, Wharton SB, Fernando M, Gelsthorpe CH, Baxter L, Ince PG, Lewis CE, Shaw PJ. Expression of vascular endothelial growth factor and its receptors in the central nervous system in amyotrophic lateral sclerosis. J Neuropathol Exp Neurol 2006;65(1):26-36.

[7] Chanakira A, Dutta R, Charboneau R, Barke R, Santilli SM, Roy S. Hypoxia differentially regulates arterial and venous smooth muscle cell proliferation via PDGFR-beta and VEGFR-2 expression. Am J Physiol Heart Circ Physiol 2012;302(5):H1173-H1184.

[8] Cheng C, Haasdijk RA, Tempel D, den Dekker WK, Chrifi I, Blonden LA, van de Kamp EH, de BM, Burgisser PE, Noorderloos A, Rens JA, ten Hagen TL, Duckers HJ. PDGF-induced migration of vascular smooth muscle cells is inhibited by heme oxygenase-1 via VEGFR2 upregulation and subsequent assembly of inactive VEGFR2/PDGFRbeta heterodimers. Arterioscler Thromb Vasc Biol 2012;32(5):1289-98.
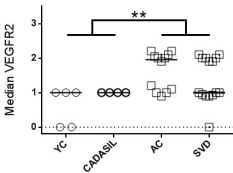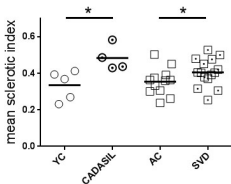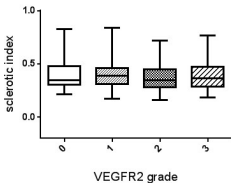
[9] Dassan P, Brown MM, Gregoire SM, Keir G, Werring DJ. Association of cerebral microbleeds in acute ischemic stroke with high serum levels of vascular endothelial growth factor. Arch Neurol 2012;69(9):1186-9.

[10] Duval M, Bedard-Goulet S, Delisle C, Gratton JP. Vascular endothelial growth factor-dependent down-regulation of Flk-1/KDR involves Cbl-mediated ubiquitination. Consequences on nitric oxide production from endothelial cells. J Biol Chem 2003;278(22):20091-7.

[11] Esiri MM, Wilcock GK, Morris JH. Neuropathological assessment of the lesions of significance in vascular dementia. J Neurol Neurosurg Psychiatry 1997;63(6):749-53.

[12] Fernando MS, Simpson JE, Matthews F, Brayne C, Lewis CE, Barber R, Kalaria RN, Forster G, Esteves F, Wharton SB, Shaw PJ, O'Brien JT, Ince PG. White matter lesions in an unselected cohort of the elderly: molecular pathology suggests origin from chronic hypoperfusion injury. Stroke 2006;37(6):1391-8.

[13] Fisher CM. The arterial lesions underlying lacunes. Acta Neuropathol 1968;12(1):1-15.

[14] Giwa MO, Williams J, Elderfield K, Jiwa NS, Bridges LR, Kalaria RN, Markus HS, Esiri MM, Hainsworth AH. Neuropathologic evidence of endothelial changes in cerebral small vessel disease. Neurology 2012;78(3):167-74.

[15] Greenberg DA, Jin K. Vascular endothelial growth factors (VEGFs) and stroke. Cell Mol Life Sci 2013;70(10):1753-61.

[16] Guo LH, Alexopoulos P, Perneczky R. Heart-type fatty acid binding protein and vascular endothelial growth factor: cerebrospinal fluid biomarker candidates for Alzheimer's disease. Eur Arch Psychiatry Clin Neurosci 2013;263(7):553-60.

[17] Hachinski V, Iadecola C, Petersen RC, Breteler MM, Nyenhuis DL, Black SE, Powers WJ, DeCarli C, Merino JG, Kalaria RN, Vinters HV, Holtzman DM, Rosenberg GA, Dichgans M, Marler JR, Leblanc GG. National Institute of Neurological Disorders and Stroke-Canadian Stroke Network vascular cognitive impairment harmonization standards. Stroke 2006;37(9):2220-41.

[18] Hainsworth AH, Oommen AT, Bridges LR. Endothelial cells and human cerebral small vessel disease. Brain Pathol 2015;25(1):44-50.

[19] Hayakawa K, Pham LD, Som AT, Lee BJ, Guo S, Lo EH, Arai K. Vascular endothelial growth factor regulates the migration of oligodendrocyte precursor cells. J Neurosci 2011;31(29):10666-70.

[20] Hogervorst E, Ribeiro HM, Molyneux A, Budge M, Smith AD. Plasma homocysteine levels, cerebrovascular risk factors, and cerebral white matter changes (leukoaraiosis) in patients with Alzheimer disease. Arch Neurol 2002;59(5):787-93.

[21] Hohman TJ, Bell SP, Jefferson AL. The role of vascular endothelial growth factor in neurodegeneration and cognitive decline: exploring interactions with biomarkers of Alzheimer disease. JAMA Neurol 2015;72(5):520-9.

[22] Ishida A, Murray J, Saito Y, Kanthou C, Benzakour O, Shibuya M, Wijelath ES. Expression of vascular endothelial growth factor receptors in smooth muscle cells. J Cell Physiol 2001;188(3):359-68.

[23] Kalaria RN, Cohen DL, Premkumar DR, Nag S, LaManna JC, Lust WD. Vascular endothelial growth factor in Alzheimer's disease and experimental cerebral ischemia. Brain Res Mol Brain Res 1998;62(1):101-5.

[24] Krum JM, Mani N, Rosenstein JM. Roles of the endogenous VEGF receptors flt-1 and flk-1 in astroglial and vascular remodeling after brain injury. Exp Neurol 2008;212(1):108-17.

[25] Lammie GA. Hypertensive cerebral small vessel disease and stroke. Brain Pathol 2002;12(3):358-70.

[26] Lammie GA, Brannan F, Slattery J, Warlow C. Nonhypertensive cerebral small-vessel disease. An autopsy study. Stroke 1997;28(11):2222-9.

[27] Medana IM, Day NP, Roberts R, Sachanonta N, Turley H, Pongponratn E, Hien TT, White NJ, Turner GD. Induction of the vascular endothelial growth factor pathway in the brain of adults with fatal falciparum malaria is a non-specific response to severe disease. Histopathology 2010;57(2):282-94.

[28] Miao Q, Paloneva T, Tuisku S, Roine S, Poyhonen M, Viitanen M, Kalimo H. Arterioles of the lenticular nucleus in CADASIL. Stroke 2006;37(9):2242-7.

[29] Murdaca J, Treins C, Monthouel-Kartmann MN, Pontier-Bres R, Kumar S, Van OE, Giorgetti-Peraldi S. Grb10 prevents Nedd4-mediated vascular endothelial growth factor receptor-2 degradation. J Biol Chem 2004;279(25):26754-61.

[30] Potente M, Gerhardt H, Carmeliet P. Basic and therapeutic aspects of angiogenesis. Cell 2011;146(6):873-87.

[31] Prins ND, Scheltens P. White matter hyperintensities, cognitive impairment and dementia: an update. Nat Rev Neurol 2015;11(3):157-65.

[32] Rahimi N, Dayanir V, Lashkari K. Receptor chimeras indicate that the vascular endothelial growth factor receptor-1 (VEGFR-1) modulates mitogenic activity of VEGFR-2 in endothelial cells. J Biol Chem 2000;275(22):16986-92.

[33] Reeson P, Tennant KA, Gerrow K, Wang J, Weiser NS, Thompson K, Lockhart KL, Holmes A, Nahirney PC, Brown CE. Delayed inhibition of VEGF signaling after stroke attenuates blood-brain barrier breakdown and improves functional recovery in a comorbidity-dependent manner. J Neurosci 2015;35(13):5128-43.

[34] Religa P, Cao R, Religa D, Xue Y, Bogdanovic N, Westaway D, Marti HH, Winblad B, Cao Y. VEGF significantly restores impaired memory behavior in Alzheimer's mice by improvement of vascular survival. Sci Rep 2013;3:2053.

[35] Rossi R, Joachim C, Geroldi C, Combrinck M, Esiri MM, Smith AD, Frisoni GB. Association between subcortical vascular disease on CT and neuropathological findings. Int J Geriatr Psychiatry 2004;19(7):690-5.

[36] Shintani Y, Takashima S, Asano Y, Kato H, Liao Y, Yamazaki S, Tsukamoto O, Seguchi O, Yamamoto H, Fukushima T, Sugahara K, Kitakaze M, Hori M. Glycosaminoglycan modification of neuropilin-1 modulates VEGFR2 signaling. EMBO J 2006;25(13):3045-55.

[37] Storkebaum E, Ruiz de AC, Meens M, Zacchigna S, Mazzone M, Vanhoutte G, Vinckier S, Miskiewicz K, Poesen K, Lambrechts D, Janssen GM, Fazzi GE, Verstreken P, Haigh J, Schiffers PM, Rohrer H, Van der Linden A, De Mey JG, Carmeliet P. Impaired autonomic regulation of resistance arteries in mice with low vascular endothelial growth factor or upon vascular endothelial growth factor trap delivery. Circulation 2010;122(3):273-81.

[38] Stowe AM, Plautz EJ, Eisner-Janowicz I, Frost SB, Barbay S, Zoubina EV, Dancause N, Taylor MD, Nudo RJ. VEGF protein associates to neurons in remote regions following cortical infarct. J Cereb Blood Flow Metab 2007;27(1):76-85.

[39] Stowe AM, Plautz EJ, Nguyen P, Frost SB, Eisner-Janowicz I, Barbay S, Dancause N, Sensarma A, Taylor MD, Zoubina EV, Nudo RJ. Neuronal HIF-1 alpha protein and VEGFR-2 immunoreactivity in functionally related motor areas following a focal M1 infarct. J Cereb Blood Flow Metab 2008;28(3):612-20.

[40] Whitman GT, DiPatre PL, Lopez IA, Liu F, Noori NE, Vinters HV, Baloh RW. Neuropathology in older people with disequilibrium of unknown cause. Neurology 1999;53(2):375-82.

[41] Wittko-Schneider IM, Schneider FT, Plate KH. Brain homeostasis: VEGF receptor 1 and 2-two unequal brothers in mind. Cell Mol Life Sci 2013;70(10):1705-25.

[42] Yamamoto Y, Ihara M, Tham C, Low RW, Slade JY, Moss T, Oakley AE, Polvikoski T, Kalaria RN. Neuropathological correlates of temporal pole white matter hyperintensities in CADASIL. Stroke 2009;40(6):2004-11.

[43] Yao JS, Zhai W, Fan Y, Lawton MT, Barbaro NM, Young WL, Yang GY. Interleukin-6 upregulates expression of KDR and stimulates proliferation of human cerebrovascular smooth muscle cells. J Cereb Blood Flow Metab 2007;27(3):510-20.

Age-dependent expression of VEGF-R2 in deep brain arteries in small vessel disease, CADASIL and healthy brains

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Supplementary Methods

Multiple labelling immunofluorescence

Paraffin sections (6 μm thickness) were processed for immunofluorescence labeling as in our previous work, see [1, 3]. Heat-induced antigen retrieval was performed using a Menarini-Biocare decloaker, (120ºC, 30 s, in citrate buffer pH 6). Non-specific binding was blocked by incubation with 3 % w/v BSA (Jackson Immunochemicals) in PBS-T for 1 hour at room temperature. Sections were incubated overnight in a humidified chamber at 4ºC with the following primary antibodies: VEGF-R2 (1:100) and α-SMA (1:200), diluted in 3 % w/v BSA in PBS-T. Sections were incubated with appropriate secondary antibodies conjugated to Alexa488 or Alexa546, diluted 1:200 in 3 % BSA in PBS-T at room temperature for 1 h. After nuclear labeling with DAPI (30 min, 0.3 µM in PBS-T) sections were mounted and photographed with a Zeiss LSM 510Meta confocal microscope. Red fluorescence was viewed with 543 nm excitation and 545-575 nm emission bandwidth. Green fluorescence was viewed with 488 nm excitation and 505-530 nm emission bandwidth. DAPI was viewed with 364 nm excitation and 385-470 nm emission bandwidth. Neighboring sections were processed identically in parallel, but with omission of primary antibodies.

Automated estimation of blood vessel density

Immunohistochemical labelling for CD34 and SMA (visualised with DAB chromogen) was carried out on neighbouring sections from the same tissue block that was used for VEGF-R2. Both CD34 and SMA revealed a fine network of mainly small vessels. TIFF images were sampled at 4x magnification at 3 locations (as in our previous work)[2]. Briefly, the sections were considered as being divided in three equal divisions, and the image sampled at the centre of each division (avoiding tissue artefacts and large vessels). TIFF images were colour-thresholded into haematoxylin and DAB components using the H-DAB macro within ImageJ software, and the DAB (brown) area fractions obtained in ImageJ. An average of the CD34 positive area fraction, and the SMA positive area fraction for the 3 images was calculated in each case.
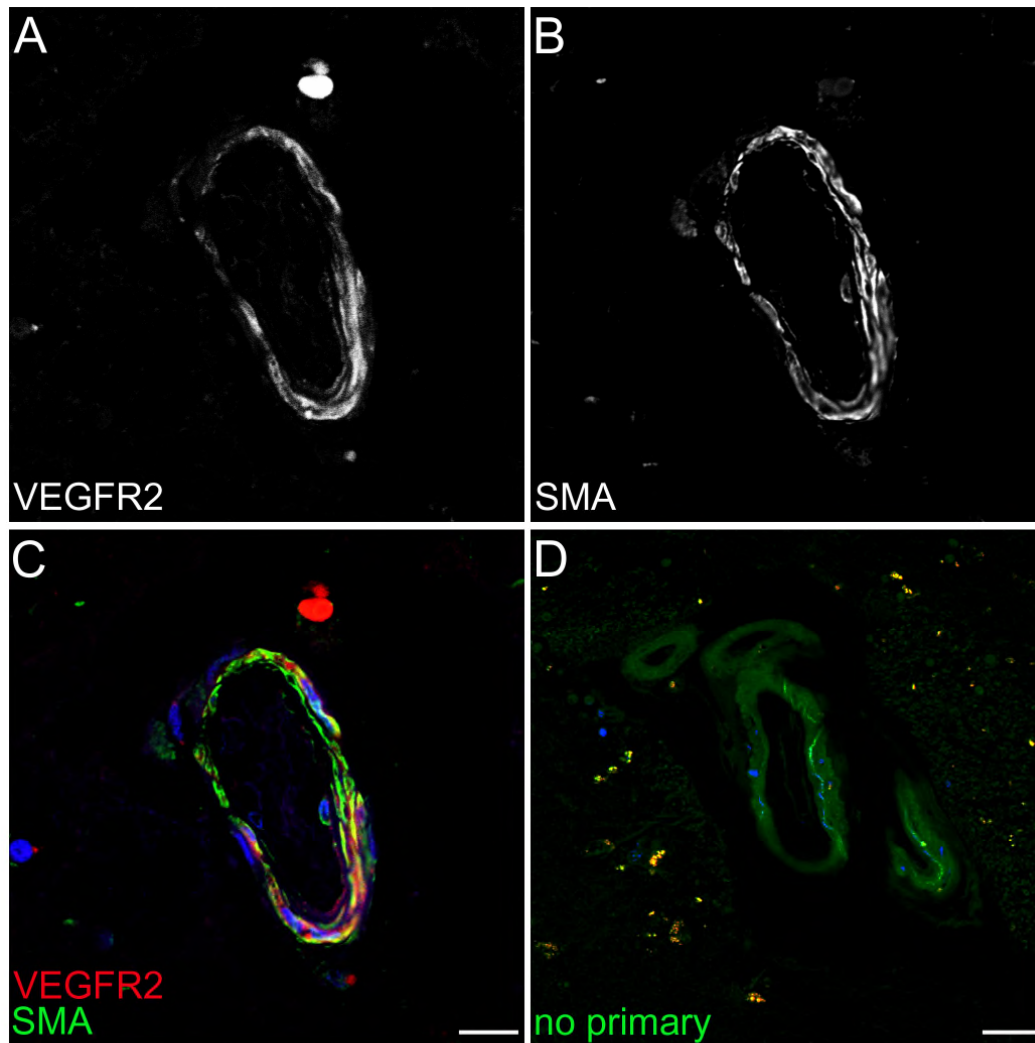
Figure S1. Confocal immunofluorescence labelling of a small artery with VEGF-R2 and the myocyte specific marker smooth muscle α-actin (SMA).

A, B: VEGF-R2 labelling in the red channel (panel A) and SMA in the green channel (B). The triple labelled image (panel C) shows clear overlap, denoted in yellow. The nuclear counterstain is DAPI (blue). D: a neighbouring section treated identically but without primary antibodies shows no cellular labelling. Some autofluorescence due to lipofuschin is seen, most evident in the red channel, and some green fluorescence from fibrous elements in the vessel wall in panel D. Scale bar: A-C: 20 microns, D: 100 microns.
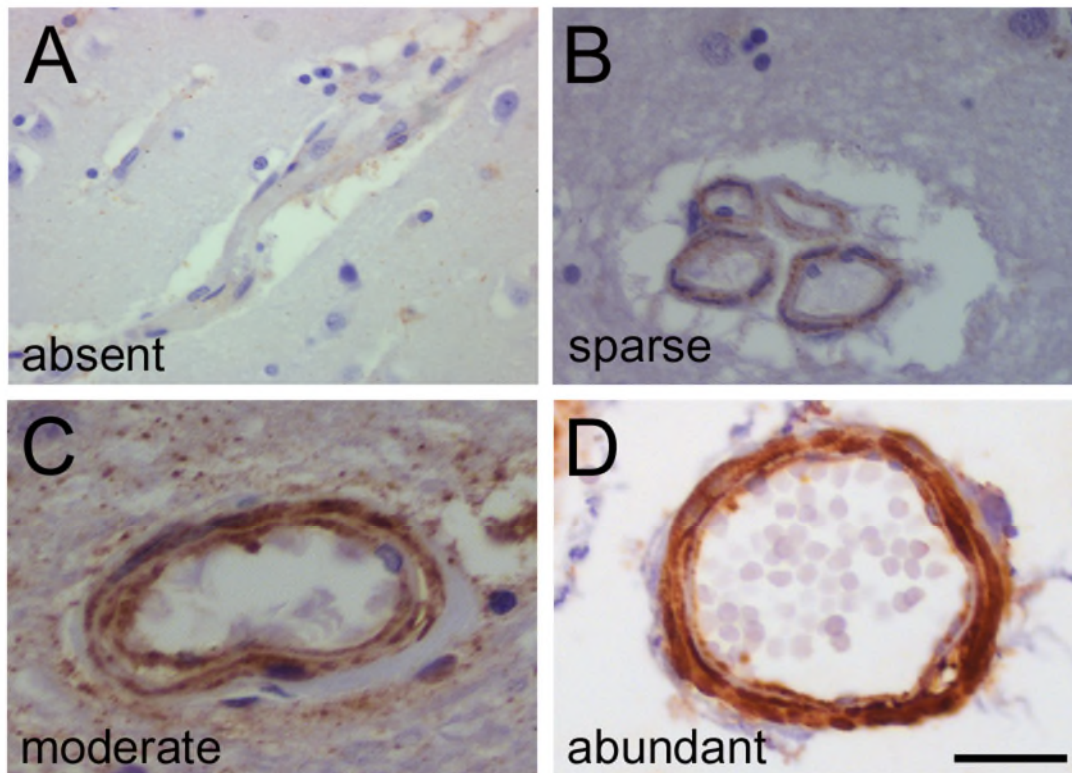
Figure S2. Examples of myocyte VEGF-R2 labelling grades in small arterial vessels.
A, myocyte labelling is absent (grade 0). B, sparse labelling (grade 1) in a convoluted arteriole. C, moderate labelling (grade 2). D, abundant labelling (grade 3). Scale bar 20 microns.

Supplementary References

[1] Andoh J, Sawyer B, Szewczyk K, Nortley M, Rossetti T, Loftus IM, Yanez-Munoz RJ, Hainsworth AH. Transgene delivery to endothelial cultures derived from porcine carotid artery ex vivo. Transl Stroke Res 2013;4(5):507-14.

[2] Bridges LR, Andoh J, Lawrence AJ, Khoong CH, Poon WW, Esiri MM, Markus HS, Hainsworth AH. Blood-brain barrier dysfunction and cerebral small vessel disease (arteriolosclerosis) in brains of older people. J Neuropathol Exp Neurol 2014;73(11):1026-33.

[3] Hainsworth AH, Bermpohl D, Webb TE, Darwish R, Fiskum G, Qiu J, McCarthy D, Moskowitz MA, Whalen MJ. Expression of cellular FLICE inhibitory proteins (cFLIP) in normal and traumatic murine and human cerebral cortex. J Cereb Blood Flow Metab 2005;25(8):1030-40.