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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 characterized by fibrosis and depletion of vascular myocytes in small penetrating arteries. Vascular endothelial growth factor (VEGF) is associated with brain aging, and Immunolabeling for vascular endothelial growth factor receptor 2 (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. Immunolabeling for VEGFR2 in deep gray 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 (Cerebral Autosomal-Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy). 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.

1. Introduction

Cerebral small vessel disease (SVD; arteriolosclerosis) is a widespread aging-related vasculopathy that causes lacunar stroke (Fisher, 1968; Lammie, 2002), diffuse white matter lesions (Prins and Scheltens, 2015; Rossi et al., 2004), and vascular cognitive impairment and dementia (Esiri et al., 1997; Hachinski et al., 2006). SVD is characterized by fibrotic thickening in the wall of small penetrating arteries, with depletion of vascular smooth muscle cells (VSMC) (Hainsworth et al., 2015; Lammie, 2002; Lammie et al., 1997). Although 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 (Potente et al., 2011; Wittko-Schneider et al., 2013). Activation of VEGFR2 in endothelial cells by the canonical ligand vascular endothelial growth factor (VEGF) is associated with angiogenesis and increased vascular permeability. Within central nervous system tissues, VEGFR2 has been reported in neurones (Boer et al., 2008; Brockington et al., 2006; Krum et al., 2008; Medana et al., 2010; Stowe et al., 2008), oligodendrocyte progenitors (Hayakawa et al., 2011), microglial cells (Fernando et al., 2006), and in VSMC within spinal cord (Brockington et al., 2006) and retinal arteries (Cheng et al., 2012). The ligand VEGF is also expressed in brain tissue (Barker et al., 2014; Kalaria et al., 1998; Stowe et al., 2007).

In adult brain, VEGF is considered to play a role in neurorepair, after cerebrovascular injury (Greenberg and Jin, 2013). After focal ischemia in cerebral cortex of nonhuman primates, VEGF and VEGFR2 are both augmented (Stowe et al., 2007, 2008), and VEGF is elevated in cerebrospinal fluid (CSF) of ischemic stroke patients(Dassan et al., 2012). A role for VEGF in age-related dementia also appears likely(Kalaria et al., 1998). 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 (Hohman et al., 2015).

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Here we observed VEGFR2 in VSMCs within small penetrating arteries in caudate-putamen of older people, a subcortical gray matter region known to develop SVD (Fisher, 1968; Lammie, 2002). 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

Postmortem 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: older than 65 year) were derived from the Oxford Project to Investigate Memory and Ageing (www.ndcn.ox.ac.uk/research/centre-prevention-stroke-dementia/resources/optima-oxford-project-to-investigate-memory-and-ageing) (Hogervorst et al., 2002). Older people with neuropathological diagnosis of moderate-severe SVD were of average age (±SD): 80.1 ± 10.7 years (range 66–99 years, n=15, 10 male:5 female). Older people without neuropathological SVD: 79.5 ± 5.8 years (68–89 years, n=12, 6 male:6 female). Younger people without brain pathology: 30.0 ± 12.3 years (male aged 11 years (M11), F26, M32, M40, F41). Cerebral Autosomal-Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL) patients: 56.5 ± 9.9 years (F44, M55, M59, and F68). This is a well-preserved tissue cohort that we have examined previously (Bridges et al., 2014; Giwa et al., 2012). All tissue samples were donated after 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, before wax embedding. Mean postmortem delay was 42 ± 23 hours (mean, SD; range 18–115 hours), and mean formalin time from postmortem examination to wax embedding was 6 ± 2 months (range: 1–9 months). All cases were free from Alzheimer's neuropathology as defined by Consortium to Establish a Registry for Alzheimer's Disease guidelines and National Institute on Aging-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 (Yamamoto et al., 2009). In all cases, endothelia were positive for cluster of differentiation CD34 immunolabeling (not shown), suggesting intact antigenicity.

2.2. Neuropathological assessment of SVD

SVD was defined on microscopic examination of standard hematoxylin and eosin sections by a registered Neuropathologist (Dr C Joachim or Margaret M. Esiri), using a semi-quantitative assessment scale (Esiri et al., 1997). 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 fibers with gliosis in white matter or loss of nerve cells and gliosis in deep gray matter in 1 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 fibers, or gliosis in white matter, or loss of nerve cells and gliosis in deep gray matter (Bridges et al., 2014; Esiri et al., 1997; Giwa et al., 2012).

2.3. Antibodies

VEGFR2 primary antibody (rabbit monoclonal; clone 55B11) was from Cell Signaling Technology, Danvers, MA, USA. 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 labeling and did not cross-react with VEGF-R1 or VEGF-R3 (Rahimi et al., 2000). CD34 antibody (clone QBend10, mouse monoclonal IgG_1) was from Novocastra-Leica Microsystems, Newcastle upon Tyne, UK. Glial fibrillary acidic protein (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 dewaxed and processed for standard immunohistochemical staining (Bridges et al., 2014; Giwa et al., 2012). After exposure to H_2O_2 (0.5 mol/L) to abolish endogenous peroxidize activity and high-pressure heat-induced antigen retrieval (0.5 minutes, 120 °C, pH 7.8), sections were blocked with bovine serum albumen (3 % w/v) in phosphate-buffered saline containing 0.1 % v/v Triton-X100 (phosphate-buffered saline containing Triton-X100 is referred to as PBT). VEGFR2 primary antibody was diluted (1:600) in PBT containing 3 % w/v bovine serum albumen and applied to sections overnight (4 °C). Other primary antibodies were applied for 60 minutes at room temperature: CD34 (1:100), Glial fibrillary acidic protein (1:10,000), and SMA (1:500). Antibody labeling was visualized using a peroxidize-conjugated secondary reagent and diaminobenzidine chromogen (Envision kit, K4065, Dako, Carpinteria, CA, USA), then counterstained with Mayer's hematoxylin. As a negative control, neighboring sections were treated without any primary antibody or with irrelevant primary antibody (rabbit anti-sheep IgG, 1:100; BD-Pharmingen).

2.5. Morphometric methods

Diaminobenzidine-labeled sections were examined on a Zeiss Axioplan-2 microscope driven by Axiovision software (version 4.7). Within each VEGFR2-labeled section, all vessels of arterial appearance with outer diameter between 20 and 200 μm, with a clear, noninflected 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, 2 blinded observers (Fiyyaz Ahmed-Jushuf; Nadim S. Jiwa) 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 2 observers differed by at most 1 category (on 65 vessels, 12.7 %), and for all these consensus was reached by discussion.

Sclerotic index (defined as 1, inner diameter and/or outer diameter) is a measure of SVD severity at the single-vessel level (Giwa et al., 2012; Hachinski et al., 2006; Lammie et al., 1997; Miao et al., 2006). 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 (Hachinski et al., 2006). Sclerotic index <0.3 corresponds to normal vessels without SVD; 0.3–0.5 to moderate SVD; and >0.5 severe SVD (Lammie et al., 1997; Miao et al., 2006; Whitman et al., 1999).