Post-mortem assessment of hypoperfusion of cerebral cortex in Alzheimer’s disease and vascular dementia

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Perfusion is reduced in the cerebral neocortex in Alzheimer’s disease. We have explored some of the mechanisms, by measurement of perfusion-sensitive and disease-related proteins in post-mortem tissue from Alzheimer’s disease, vascular dementia and age-matched control brains. To distinguish physiological from pathological reduction in perfusion (i.e. reduction exceeding the decline in metabolic demand), we measured the concentration of vascular endothelial growth factor (VEGF), a protein induced under conditions of tissue hypoxia through the actions of hypoxia-inducible factors, and the myelin associated glycoprotein to proteolipid protein 1 (MAG:PLP1) ratio, which declines in chronically hypoperfused brain tissue. To evaluate possible mechanisms of hypoperfusion, we also measured the levels of amyloid-β₄₀, amyloid-β₄₂, von Willebrand factor (VWF; a measure of microvascular density) and the potent vasoconstrictor endothelin 1 (EDN1); we assayed the activity of angiotensin I converting enzyme (ACE), which catalyses the production of another potent vasoconstrictor, angiotensin II; and we scored the severity of arteriolar sclerotic small vessel disease and cerebral amyloid angiopathy, and determined the Braak tangle stage. VEGF was markedly increased in frontal and parahippocampal cortex in Alzheimer’s disease but only slightly and not significantly in vascular dementia. In frontal cortex the MAG:PLP1 ratio was significantly reduced in Alzheimer’s disease and even more so in vascular dementia. VEGF but not MAG:PLP1 increased with Alzheimer’s disease severity, as measured by Braak tangle stage, and correlated with amyloid-β₄₂ and amyloid-β₄₀ but not amyloid-β₄₀. Although MAG:PLP1 tended to be lowest in cortex from patients with severe small vessel disease or cerebral amyloid angiopathy, neither VEGF nor MAG:PLP1 correlated significantly with the severity of structural vascular pathology (small vessel disease, cerebral amyloid angiopathy or VWF). However, MAG:PLP1 showed a significant negative correlation with the level of EDN1, which we previously showed to be elevated in the cerebral cortex Alzheimer’s disease. These finding are in contrast with the previously demonstrated reduction in EDN1, and positive correlation with MAG:PLP1, in the hypoperfused white matter in Alzheimer’s disease. The decline in MAG:PLP1 strongly suggests pathological hypoperfusion of the frontal cortex in Alzheimer’s disease. Although severe small vessel disease or cerebral amyloid angiopathy may contribute in some cases, abnormal vascular contractility mediated by EDN1 is likely to be a more important overall contributor. Both amyloid-β accumulation and hypoperfusion are likely to cause the upregulation of VEGF.

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Introduction

Abnormalities broadly described as ‘cerebrovascular’ are common in Alzheimer’s disease (Kalaria, 2000). As many as 60% of patients were reported to have ischaemic white matter damage (Brun and Englund, 1986a, b; Englund et al., 1988; Brun et al., 1990; Englund and Brun, 1990; Kalaria, 2000), attributed to arteriosclerotic small vessel disease. Cerebral angiopathy, characterized by deposition of amyloid-β in vessel walls, is also present in >90% of patients with Alzheimer’s disease (Esiri and Wilcock, 1986; Ellis et al., 1996; Love et al., 2003). There is increasing recognition that alterations to endothelial and smooth muscle cells, pericytes and the extracellular matrix of intracerebral blood vessels are not simply a consequence of the disease; by disrupting the blood–brain barrier and the transport of solutes and macromolecules along and across the walls of blood vessels, these alterations contribute to the accumulation of amyloid-β and several other potentially toxic metabolites (Weller et al., 2008; Hawkes et al., 2011, 2012; Zlokovic, 2011; Sagare et al., 2013).

Brain ischaemia is the defining pathological process in most patients with vascular dementia but cerebral blood flow is also reduced in Alzheimer’s disease (Sharp et al., 1986; Jagust et al., 1987; Schuff et al., 2009) and probably contributes to the cognitive impairment (DeKosky et al., 1990; Imran et al., 1999; Shiraishi et al., 2004). In mouse models of amyloid-β accumulation, reduced cerebral blood flow occurs well before any pathological or neurological abnormalities (Niwa et al., 2002a, b; Iadecola, 2004). Reduced cerebral blood flow also anticipates the development of dementia in Alzheimer’s disease (Ruitenbergen et al., 2005). There is increasing evidence that ischaemia is not only an additive cause of brain damage in Alzheimer’s disease but contributes directly to the underlying neurodegenerative disease processes (reviewed in Chui et al., 2012). Ischaemia in animal models, or its in vitro stimulation by combined deprivation of oxygen and glucose, is associated with increased production of amyloid-β in animal and cell culture models (Sun et al., 2006; Zhang et al., 2007; Guglielmo et al., 2009; Li et al., 2009; Zhiyou et al., 2009). However, not all studies have supported a link between cerebrovascular abnormalities and Alzheimer’s disease (Schneider et al., 2004; Chui et al., 2012; Richardson et al., 2012), and it remains unclear to what extent the reduction in cerebral blood flow in Alzheimer’s disease is a physiological response to reduced metabolic activity, and to what extent pathological, resulting in inadequate tissue oxygenation.

Structural vascular abnormalities such as arteriosclerosis and cerebral amyloid angiopathy have been a major focus of research in dementia and probably contribute to ischaemic damage in Alzheimer’s disease. However, alterations in vascular contractility that are not caused by structural vessel abnormalities are also likely to contribute (Love and Miners, 2015). Amyloid-β peptides were reported to enhance vasoconstriction, reduce cerebral blood flow and impair both functional hyperaemia and cerebral autoregulation in mice transgenic for mutant human amyloid-β precursor protein (APP) (Niwa et al., 2001, 2002a, b). We showed that the level of endothelin 1 (EDN1), a potent vasoconstrictor, is elevated in the cerebral cortex in Alzheimer’s disease (Palmer et al., 2012, 2013) and that this is likely to be a consequence of upregulation of endothelin-converting enzymes 1 and 2 (ECE1 and ECE2), which are themselves induced by amyloid-β (Palmer et al., 2009, 2010). The level and activity of angiotensin-converting enzyme (ACE), the rate-limiting enzyme for the production of angiotensin II (another potent vasoconstrictor), were also significantly increased in Alzheimer’s disease brain tissue and were upregulated by exposure to aggregated amyloid-β_{42} in vitro (Miners et al., 2008, 2009). ECE1, ECE2 (Eckman et al., 2001) and ACE degrade amyloid-β (Hu et al., 2001) and are probably upregulated in Alzheimer’s disease as a side-effect of the accumulation of excessive substrate, in the form of amyloid-β.

Vascular endothelial growth factor (VEGF) is a potent pro-angiogenic (Ferrara et al., 1995; Carmeliet and Collen, 1997) and neuroprotective factor (reviewed in Stoerkbaum et al., 2004; Greenberg and Jin, 2005) that is abundant in neurons and astroglia. It is induced under conditions of tissue hypoxia (Liu et al., 1995; Shweiki et al., 1995; Pham et al., 2002) through the actions of hypoxia-inducible factors, the principal sensors of cellular oxygenation. Upregulation of VEGF is a physiological response to tissue hypoxia, a means of increasing vascularity and restoring blood flow. In post-mortem brain tissue, we showed a highly significant correlation between VEGF concentration and the severity of small vessel disease-associated white matter hypoperfusion (Barker et al., 2014), as indicated by reduction in the ratio between the concentrations of myelin-associated glycoprotein (MAG), which is highly sensitive to ischaemia, and of proteolipid protein 1 (PLP1) that is relatively resistant (Barker et al., 2013). VEGF immunoreactivity is increased in brain tissue in Alzheimer’s disease (Kalaria et al., 1998). In the CSF, too, VEGF is elevated in Alzheimer’s disease, even more so than in vascular dementia (Tarkowski et al., 2002) (in contrast, VEGF is reduced in the serum in Alzheimer’s disease; Mateo et al., 2007). There is conflicting evidence as to whether amyloid-β upregulates VEGF directly. Chiarini et al. (2010) reported that amyloid-β_{42} stimulates...
production of VEGF by normoxic human cerebral astrocytes in vitro. However, in another study (Park and Chae, 2007), exposure of HN33 neuronal cells, transformed human endothelial cells (ECV304), primary astrocytes and NIH-3T3 cells to 2 μM or 10 μM amyloid-β42 had no effect on VEGF synthesis.

In the present study, we have measured the level of VEGF and the MAG:PLP1 ratio in human post-mortem brain tissue in Alzheimer’s disease, vascular dementia and age-matched controls and have investigated the relationship between these two indices of tissue perfusion and a range of markers of Alzheimer’s disease severity, microvascular pathology and vascular dysfunction. Our findings provide evidence of a pathological decline in oxygenation of cerebral cortex in Alzheimer’s disease that is independent of structural vascular disease but associated with elevation in the concentration of the potent vasoconstrictor EDN1.

Materials and methods

Study cohort

Brain tissue was obtained from the South West Dementia Brain Bank, University of Bristol, UK, with local Research Ethics Committee approval. The brains had been separated mid-sagittally: the left hemisphere sliced and frozen at −80°C and the right hemisphere fixed in formalin for paraffin histology and detailed neuropathological examination.

The biochemical analyses in the present study were on cortical tissue samples from 20 patients with Alzheimer’s disease, 17 patients with vascular dementia and 20 age-matched controls. The demographic and pathological information is shown in Table 1 and the MRC UK Brain Banks Network database identifiers are listed in Supplementary Table 1. The diagnosis of Alzheimer’s disease required a diagnosis of dementia by experienced clinicians using standard criteria as specified in the Diagnostic and Statistical Manual of Mental Disorders Fourth Edition (DSM-IV), and a high likelihood that Alzheimer’s disease neuropathological changes were sufficient explanation for the dementia, according to National Institute on Aging-Alzheimer’s Association guidelines (Montine et al., 2012). The vascular dementia cases had a clinical history of dementia, no more than occasional neuritic plaques, a Braak tangle stage of III or less, histopathological evidence of multiple infarcts/ischaemic lesions, moderate to few plaques, a Braak tangle stage of III or less, histopathological evidence of other disease likely to cause dementia. The normal controls had no history of dementia, few or no neuritic plaques, and no other neuropathological abnormalities.

Small vessel disease samples in paraffin sections of right frontal and temporal lobe were scored on a four-point semi-quantitative scale (Barker et al., 2013) according to the extent of thickening of the arteriolar walls and associated narrowing of vessel lumens: 0 = normal vessel wall thickness, 1 = slightly increased thickness, 2 = moderately increased thickness, and 3 = markedly increased thickness such that for many arterioles the diameter of the lumen was <50% of the outer diameter of the blood vessel. As a small vessel disease score or 0 applied to relatively few cases, and cases with scores of 0 or 1 were grouped together for analysis. Cerebral amyloid angiopathy in the right parietal lobe had been previously graded (Chalmers et al., 2003) using a method based on that of Olichney et al. (1996) in which a score of 0 corresponded to vessels devoid of amyloid, a score of 1 to scattered deposition of amyloid in a few leptomeningeal or cortical blood vessels, a score of 2 to circumferential deposition of amyloid in several vessels, and a score of 3 to severe and widespread cerebral amyloid angiopathy.

### Table 1 Cases studied

<table>
<thead>
<tr>
<th></th>
<th>Age at death&lt;sup&gt;a&lt;/sup&gt; (y)</th>
<th>Gender&lt;sup&gt;a&lt;/sup&gt; (M:F)</th>
<th>Post-mortem delay&lt;sup&gt;b&lt;/sup&gt; (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 20)</td>
<td>81 ± 10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8:12</td>
<td>33 ± 17&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Alzheimer’s disease (n = 20)</td>
<td>80 ± 7</td>
<td>9:11</td>
<td>33 ± 19&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Vascular dementia (n = 17)</td>
<td>83 ± 8</td>
<td>8:9</td>
<td>44 ± 17&lt;sup&gt;a&lt;/sup&gt;</td>
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<sup>a</sup>For a full list of all cases with MRC brain bank identifiers, see Supplementary Table 1.
<sup>b</sup>Mean ± SD.

Tissue preparation

Approximately 200 mg of frozen samples of frontal (Brodmann area 8), parahippocampal (Brodmann area 36) and cingulate cortex (Brodmann area 24) and thalamus were dissected and homogenized in SDS lysis buffer (5 mM NaCl, 1 M Tris-HCl pH 7.6, 1 mM phenylmethylsulphonyl fluoride, 1.7 mg/ml aprotinin and 10% SDS) in a mechanical Precellys 24 homogenizer (Stretton Scientific) with 2.3 mm ceramic beads (Biospec). The homogenates were spun at 13000g for 15 min at 4°C and the supernatants removed and stored at −80°C. Total protein concentration was determined using Total Protein Kit (Sigma Aldrich) following the manufacturer’s guidelines.

VEGF sandwich ELISA

VEGF protein level was measured using Human VEGF Quantikine ELISA kit (R&D Systems) according to the manufacturer’s guidelines. The ELISA used a monoclonal mouse VEGF antibody as a capture antibody and a polyclonal biotinylated VEGF detection antibody. Tissue samples (1.0 mg/ml total protein) were added in duplicate and incubated for 2 h at room temperature with constant shaking. VEGF concentration was interpolated from a standard curve generated by serial dilution of human recombinant VEGF (31.25–2000 pg/ml). Absorbance was read at 450 nm in a FLUOstar Optima plate reader.

In pilot studies, we confirmed the post-mortem stability of VEGF, by comparing measurements made on aliquots of adjacent occipital cortex from one control and one Alzheimer’s disease brain (n = 2) with relatively short post-mortem delays (6–10 h). We incubated the samples for up to 72 h at either room temperature or 4°C. The protein levels did not change significantly over 72 h, even at room temperature (Supplementary Fig. 1).
MAG and PLP1 ELISA

MAG level was determined as previously described (Barker et al., 2013, 2014; Miners et al., 2014). Brain tissue homogenates were diluted 1:10 in phosphate-buffered saline (PBS), added in duplicate to clear 96-well microplates (Fisher Scientific), left for 2 h at room temperature with constant shaking, washed five times in PBS/0.01% Tween-20, blocked in 1% bovino serum albumin/PBS for 2 h at room temperature and washed again. Mouse monoclonal anti-MAG (Abcam) diluted to 0.5 μg/ml was added for 2 h at room temperature. The plates were then washed and incubated for 20 min at room temperature with biotinylated anti-mouse secondary antibody (Vector Labs) diluted 1:500. After another wash the wells were incubated with streptavidin-horseradish peroxidase (1:500) (R&D systems). Chromogenic substrate (TMBS substrate, R&D systems) was added in the dark for 30 min and the reaction then stopped with 2 N sulphuric acid. The absorbance was read at 450 nm in a FLUOstar Optima plate reader. MAG concentration was determined by interpolation against a standard curve generated by serial dilution (6.25 to 400 ng/ml) of recombinant human MAG (Abnova). We previously demonstrated that MAG is stable under conditions of simulated post-mortem delay for up to 72 h at 4°C or room temperature (Barker et al., 2013).

PLP1 measurements were made using a commercially available myelin PLP1 sandwich ELISA (cat no SEA417Hu, Uscn) following the manufacturer’s guidelines. PLP1 concentration in brain tissue homogenates (total protein 17.5 μg in PBS) was interpolated from a standard curve generated by serial dilution of recombinant human PLP1 (10–0.156 ng/ml). We previously demonstrated that PLP1 is stable under conditions of simulated post-mortem delay for up to 72 h at 4°C or room temperature (Barker et al., 2013).

Amyloid-β40 and amyloid-β42 sandwich ELISA

Tissue sampling and the preparation of soluble and insoluble (guanidine-extractable) fractions for amyloid-β measurement were reported previously (van Helmond et al., 2009; 2010a, b). Amyloid-β40 was measured by sandwich ELISA, as described (Barua et al., 2012). High-binding Costar 96-well plates (R&D Systems) were coated with mouse anti-human amyloid-β (2 μg/ml) (clone 6E10, raised against amino acids 1–16; Covance) in PBS and incubated overnight at room temperature. After five washes with PBS containing 0.05% Tween-20, the plates were blocked with 300 μl protein-free PBS blocking buffer (Thermo Fisher Scientific) for 2 h at room temperature. After a further five washes, samples of brain homogenate (diluted 1:49 for guanidine extracts and 1:3 for soluble extracts) or recombinant human amyloid-β40 (Sigma Aldrich) diluted in PBS containing 1% 1,10 phenanthroline (Sigma Aldrich) to prevent degradation of amyloid-β (Qiu et al., 1997), were incubated for 2 h at room temperature on a rocking platform. After another wash, the plates were incubated for 2 h at room temperature with mouse anti-human amyloid-β40 (1 μg/ml) (11A50-B10; Covance) that had been biotinylated using Lightning-Link® Biotinylation Kit (Innova Biosciences) according to the manufacturer’s guidelines. After further washes, streptavidin-horseradish peroxidase (R&D Systems) diluted 1:200 was added to each well for 1 h at room temperature before washing and incubation with substrate solution (TMB; R&D Systems) for 30 min in the dark. The reaction was stopped with 2 N sulphuric acid (R&D Systems) and the optical density for each well read at 450 nm in a FLUOstar plate reader (BMG Labtech).

For amyloid-β42 measurement, the procedure was as outlined above with the following modifications: mouse anti-human amyloid-β42 (1 μg/ml) (12F4; Covance) that was biotinylated using Lightning-Link® Biotinylation Kit (Innova Biosciences) according to the manufacturer’s guidelines, was used for the detection step and recombinant human amyloid-β42 (Sigma Aldrich) for the standards.

Amyloid-β40 and amyloid-β42 levels in each brain tissue sample were determined by interpolation against standard curves generated by serial dilution of recombinant human amyloid-β40 and amyloid-β42 respectively. Each assay was performed in duplicate and the mean calculated. The amyloid-β40 ELISA did not show any cross-reactivity with amyloid-β42, nor did the amyloid-β42 ELISA with amyloid-β40.

Von Willebrand factor dot blot

Von Willebrand factor (VWF) level was determined by dot blot analysis using a polyclonal rabbit anti-human VWF antibody, as previously described (Ashby et al., 2010). Samples were diluted in Tris-buffered saline (TBS; 1 in 800) and blotted onto nitrocellulose membrane (GE Healthcare) for 1 h at room temperature. The membrane was blocked in 5% non-fat dried milk protein (NFDMP) in TBS at 4°C overnight, washed, and then incubated for 1 h with polyclonal rabbit anti-human VWF, (0.3 μg/ml) (Dako) at room temperature with agitation. After washing, the membrane was incubated with anti-rabbit peroxidase-conjugated secondary antibody (Vector Laboratories) in 5% NFDMP diluted in 0.3% TBS-T for 1 h at room temperature with agitation. The membrane was washed and then developed with chemiluminescent ECL substrate (Millipore) according to the manufacturer’s guidelines. ImageJ was used to measure the integrated density of each sample. Serial dilutions of a standard reference brain tissue homogenate were used to adjust for any blot-to-blot variation. We previously demonstrated that VWF is stable under conditions of simulated post-mortem delay for up to 72 h at 4°C or room temperature (Ashby et al., 2010) and that VWF level, measured by dot blot, is an excellent measure of microvessel density (Barker et al., 2014; Miners et al., 2014).

EDN1 sandwich ELISA

EDN1 level was measured in brain tissue homogenates by use of the QuantiGlo® Chemiluminescent sandwich ELISA kit for human EDN1 (R&D Systems) following the manufacturer’s guidelines, as described previously (Palmer et al., 2012; Miners et al., 2014). Samples containing 1 mg/ml total protein were added in duplicate and incubated for 1.5 h at room temperature with agitation. Luminescence was read in a FLUOstar Optima plate reader. EDN1 concentration was interpolated from a standard curve generated by serial dilutions of recombinant human EDN1 (0.34–250 pg/ml).
Immunocapture-based fluorogenic ACE activity assay

ACE enzyme activity was measured in brain tissue homogenates by immunocapture-based activity assay as previously described (Miners et al., 2014). An ACE antibody (R&D systems, UK) diluted to 5 ng/ml in PBS was coated onto a black Fluoronunc plate overnight, then blocked in 1% BSA/PBS for 2 h at room temperature before tissue homogenates (1 mg/ml in PBS) or serial dilutions of recombinant human ACE (2500–39 pg/ml) were added in duplicate to the wells and incubated for 2 h at room temperature with constant shaking. Fluorogenic peptide substrate (ES005, R&D systems) diluted in activity assay buffer (100 mM Tris-HCl pH 7.5, 50 mM NaCl and 10 μM ZnCl₂) was added and the plate was incubated for 3 h at 37 °C in the dark, and fluorescence measured with excitation at 320 nm and excitation at 405 nm. To determine ACE-specific enzyme activity we subtracted the fluorescent signal after inhibition by captopril from that in the paired uninhibited wells. Each assay was repeated twice and the mean determined. ACE activity was interpolated from a standard curve produced by serial dilutions (39–2500 pg/ml) of recombinant human ACE (R&D systems).

Statistical analysis

Unpaired two-tailed t-tests, ANOVA with Bonferroni post hoc analysis, or Kruskall-Wallis test with Dunn’s post hoc multiple comparison test was used for comparisons between groups, and Pearson’s or Spearman’s test to assess linear or rank order correlation, as appropriate, with the help of SPSS version 16 (SPSS, Chicago) and GraphPad Prism version 6 (GraphPad Software). P-values < 0.05 were considered statistically significant.

Results

VEGF level is increased in Alzheimer’s disease

VEGF protein level was significantly elevated (~2-fold) in Alzheimer’s disease compared to age-matched controls in the frontal (Fig. 1A) and parahippocampal cortex (Fig. 1B) and the increase approached statistical significance in the cingulate cortex (Fig. 1B). Although mean VEGF level in vascular dementia was above mean control values in all three regions, the increase was less marked than that in Alzheimer’s disease and did not reach statistical significance. VEGF level was unchanged in the thalamus (an area with little amyloid pathology) in Alzheimer’s disease or vascular dementia (Fig. 1D).

MAG:PLP1 is decreased in Alzheimer’s disease and vascular dementia

We previously reported that the MAG:PLP1 ratio is a robust post-mortem marker of ante-mortem hypoperfusion of human cerebral white matter (Barker et al., 2013, 2014), the ratio falling as the severity of small vessel disease in the white matter increases. However, the cerebral cortex also contains myelinated fibres, and we recently showed that MAG is sensitive to reduced vascularity of the occipital cortex in dementia with Lewy bodies (Miners et al., 2014). We therefore measured MAG:PLP1 in the frontal cortex, the region that we had found to have the most marked increase in VEGF in Alzheimer’s disease.

The MAG:PLP1 ratio was significantly reduced in Alzheimer’s disease and further reduced in vascular dementia (Fig. 2A), indicating pathological hypoperfusion of the cortex in both diseases. There was a non-significant negative relationship between MAG:PLP1 and VEGF (Fig. 2B), suggesting a contributory rather than a dominant role for cortical hypoperfusion in the increase in VEGF in Alzheimer’s disease.

Increase in VEGF is related to disease severity in Alzheimer’s disease

To assess whether VEGF level increased in relation to Alzheimer’s disease severity, we analysed a combined cohort of Alzheimer’s disease and control brains and stratified the brains according to Braak tangle stage: stages 0–II, III–IV, and V–VI. VEGF level in frontal cortex was significantly higher in the Braak stage V–VI than the 0–II group. It was also higher, but not significantly so, in the Braak stage III–IV than the 0–II group (Fig. 3A). Spearman correlation analysis revealed a significant positive relationship between VEGF level and Braak stage (P = 0.0036). A non-significant trend towards increased VEGF in relation to disease severity was also observed in parahippocampal cortex (data not shown). VEGF level in the cingulate cortex and thalamus did not alter with Braak stage (data not shown).

We examined the relationship between VEGF and the levels of amyloid-β₄₀ and amyloid-β₄₂ within the same tissue homogenates from the frontal cortex. VEGF correlated strongly with insoluble (guanidine-HCl-extractable) amyloid-β₄₂ (Fig. 3B) and insoluble amyloid-β₄₂: amyloid-β₄₀ (Fig. 3C) but not with insoluble amyloid-β₄₀ (Fig. 3D). VEGF level did not correlate with levels of soluble amyloid-β₄₀ or amyloid-β₄₂ (data not shown). VEGF level did not correlate with any of these isoforms or fractions of amyloid-β in parahippocampal or cingulate cortex or thalamus (data not shown).

Decrease in MAG:PLP1 is not related to disease severity in Alzheimer’s disease

The MAG:PLP1 ratio decreased with Braak tangle stage but not significantly, and varied considerably within each stage (Supplementary Fig. 2A). The ratio did not correlate with the level of amyloid-β₄₂ (Supplementary Fig. 2B),
amyloid-β_{42}: amyloid-β_{40} (Supplementary Fig. 2C) or amyloid-β_{40} (Supplementary Fig. 2D) in the insoluble fraction of homogenates of frontal cortex, although there were weak negative relationships between MAG:PLP1 and amyloid-β. MAG:PLP1 did not correlate with levels of soluble amyloid-β_{40} or amyloid-β_{42} (data not shown).

**Cortical VWF, a marker of microvessel density, is normal in Alzheimer’s disease but correlates with VEGF**

To try to establish the cause of hypoperfusion of cerebral cortex in Alzheimer’s disease, we next looked at several structural vascular abnormalities that we thought might contribute. The first possibility we considered was reduced microvessel density. To assess this we measured VWF, which we showed previously to correlate closely with morphological measurement of microvessel density (Barker et al., 2014; Miners et al., 2014). VWF level was slightly

![Figure 1 Regional, disease-specific increase in VEGF in Alzheimer's disease.](image)
lower in the frontal and parahippocampal cortex in Alzheimer’s disease but the difference between the Alzheimer’s disease and control groups was not significant in any of the four regions examined (Fig 4). However, when we looked at the relationship between VEGF and VWF across the groups, we observed a significant positive correlation between VEGF and VWF in the parahippocampal cortex and thalamus and a trend in the same direction in the cingulate cortex. We did not observe a correlation in the frontal cortex.

A positive association was observed between VWF and MAG:PLP1 but this did not reach significance ($P = 0.35$, $r = 0.07$) (Supplementary Fig. 3).

**Increase in VEGF and reduction in MAG:PLP1 do not correlate with small vessel disease or cerebral amyloid angiopathy**

We next examined whether the changes in MAG:PLP1 or VEGF in Alzheimer’s disease were attributable to small vessel disease or cerebral amyloid angiopathy. The severity of small vessel disease, as measured by the small vessel disease scores in the frontal lobe, varied significantly between Alzheimer’s disease, vascular dementia and control groups (Kruskall-Wallis test, $P = 0.0007$) (Fig. 5A). Post hoc pairwise comparisons between groups by Dunn’s test showed that the severity of small vessel disease was significantly greater in vascular dementia than in controls or Alzheimer’s disease but did not differ significantly between Alzheimer’s disease and controls.

As might be expected, the cerebral amyloid angiopathy scores in the frontal lobe also differed significantly between the three groups ($P < 0.0002$) (Fig. 5B). Post hoc pairwise comparisons using Dunn’s test showed the severity of cerebral amyloid angiopathy to be significantly greater in Alzheimer’s disease than in controls or vascular dementia ($P < 0.05$) but not to differ significantly between vascular dementia and controls.

However, when we combined the three groups and examined the relationships between overall small vessel disease or cerebral amyloid angiopathy scores and VEGF or MAG:PLP1 in the frontal, cingulate or parahippocampal cortex or the thalamus, we found that VEGF level did not vary significantly in relation to the severity of small vessel disease or cerebral amyloid angiopathy (Supplementary Fig. 4). The MAG:PLP1 ratio was lowest in frontal cortex from brains with the most severe small vessel disease and lower in those with than without cerebral amyloid angiopathy (Supplementary Fig. 5). However, pairwise comparisons did not reveal significant difference in MAG:PLP1 when samples were stratified according to small vessel disease or cerebral amyloid angiopathy score. MAG:PLP1 did not correlate with cerebral amyloid angiopathy score but there was a negative correlation between...
MAG:PLP1 and small vessel disease score that approached significance (Spearman’s test $P = 0.065$).

**MAG:PLP1 correlates inversely with EDN1 level but not significantly with ACE activity**

We previously reported that the level of the vasoconstrictor EDN1 (Palmer et al., 2012) and the level and activity of ACE (which catalyses the production of the vasoconstrictor, angiotensin II) (Miners et al., 2008, 2009) were increased in post-mortem brain tissue from patients with Alzheimer’s disease. We measured EDN1 level and ACE activity in the same samples of midfrontal cortex that we used for measuring VEGF and MAG:PLP1. VEGF did not vary significantly with either EDN1 level or ACE activity (data not shown). The MAG:PLP1 ratio did, however, correlate inversely with EDN1 level (Fig. 6A) although not ACE activity (Fig. 6B).

**Discussion**

In this study, we have explored some of the mechanisms underlying the hypoperfusion of the cerebral cortex in Alzheimer’s disease. We showed that VEGF level is elevated in the neocortex Alzheimer’s disease, but although VEGF level is a useful marker of hypoperfusion of the white matter, correlating closely with severity of small vessel disease and inversely with the MAG:PLP1 ratio (Barker et al., 2013, 2014), in the neocortex the level correlated more strongly with markers of Alzheimer’s disease severity—Braak tangle stage, amyloid-$\beta_{42}$ level, and the ratio of amyloid-$\beta_{42}$:amyloid-$\beta_{40}$—than with structural or functional vascular abnormalities (small vessel disease, cerebral amyloid angiopathy, EDN1 level or ACE activity), apart from a positive correlation with the level of VWF, a measure of microvascular density. However, in both Alzheimer’s disease and vascular dementia we also demonstrated a significant reduction in the ratio of MAG:PLP1; in Alzheimer’s disease this did not correlate significantly with structural vascular pathology but showed a significant negative correlation with EDN1 level, suggesting that the elevated EDN1 in Alzheimer’s disease (Palmer et al., 2012) probably contributes to the hypoperfusion of the neocortex.

An increase in VEGF-positive perivascular astrocytes was demonstrated immunohistochemically in brain tissue from Alzheimer’s disease patients (Kalaria et al., 1998) and VEGF was shown to be increased in the CSF in Alzheimer’s disease (Tarkowski et al., 2002). We found that the increase in VEGF level in homogenates of frontal cortex was directly related to the progression of disease (as indicated by Braak tangle stage), as well as the amount of insoluble amyloid-$\beta_{42}$ (the main constituent of amyloid-$\beta$ plaques) in those same homogenates. VEGF level was not elevated in the thalamus, which accumulates much less amyloid-$\beta$ plaque than the cerebral cortex, and in the frontal cortex the VEGF level correlated strongly with the amyloid-$\beta_{42}$ level and the amyloid-$\beta_{42}$:amyloid-$\beta_{40}$ ratio. These findings are consistent with the reported induction of VEGF by exposure to amyloid-$\beta$ in vitro (Chiarini et al., 2010), the immunohistochemical detection of VEGF within amyloid-$\beta$ plaques (perhaps reflecting sequestration of VEGF) and the high-affinity binding of VEGF to amyloid-$\beta$ in vitro (Yang et al., 2004).
Our second approach to assessing the severity and exploring mechanisms of cortical hypoperfusion in Alzheimer’s disease was based on measurement of the relative concentrations of MAG, which has to be transported from the oligodendrocyte cell body to the adaxonal part of the myelin sheath and is highly sensitive to tissue hypoxia, and PLP1, which is distributed throughout the myelin sheath and more resistant to hypoxia. We showed previously that the MAG:PLP1 ratio is a robust marker of white matter hypoperfusion in human post-mortem brain tissue (Barker et al., 2013). The MAG:PLP1 ratio was significantly reduced in the cerebral cortex in both Alzheimer’s disease and vascular dementia, indicating that the reduction in perfusion of the cerebral cortex in both diseases is not simply a physiological adaptation of blood flow to match reduced metabolic demand. These findings are as would be expected in vascular dementia, but this is the first time that pathological hypoperfusion of cerebral cortex has been demonstrated by biochemical analysis of human brain tissue in Alzheimer’s disease. Our findings are in keeping with in vivo studies of cerebral oxygen extraction in Alzheimer’s disease. Functional MRI in patients with a clinical diagnosis of Alzheimer’s disease (Tohgi et al., 1998) revealed not only a decrease in cerebral perfusion in relation to that in controls, but also an increase in the mean regional oxygen extraction fraction in all regions of cortex and white matter examined. If the hypoperfusion were commensurate with the decrease in oxygen requirement, the regional oxygen extraction fraction would have been unchanged or even reduced. Similarly, spatially-resolved near infrared spectroscopy in patients with amnestic mild cognitive impairment (i.e. at high risk of developing Alzheimer’s disease), demonstrated significant reduction in the tissue oxygenation fraction (intravascular oxygenated-total haemoglobin) in the frontal cortex, both at rest and after a sit-stand manoeuvre (Tarumi et al., 2014). Thus neuroimaging data support our biochemical evidence that cortical hypoperfusion in Alzheimer’s disease exceeds the reduction in metabolic demand for oxygen and is therefore pathological.

The decline in MAG:PLP1 and elevation of VEGF in Alzheimer’s disease were independent of the severity of structural abnormalities of the cerebral vasculature in the form of small vessel disease or cerebral amyloid angiopathy. This is not to say that severe small vessel disease or cerebral amyloid angiopathy do not reduce cortical perfusion in some patients; rather, that this is not the principle cause of hypoperfusion in most patients. Microvessel density, as indicated by VWF level, tended to be reduced, although not significantly, in the frontal and parahippocampal cortex in Alzheimer’s disease. An increase in VEGF might have been expected to increase capillary density in Alzheimer’s disease, and VEGF did correlate with VWF level in some regions (parahippocampal cortex and thalamus) when the cohorts were combined. The binding and sequestration of VEGF by amyloid-β42 in plaques may limit its availability, and contribute to the lack of significant increase in VWF in Alzheimer’s disease. In addition, amyloid-β42 was shown to bind to the VEGF receptor 2 molecule, preventing VEGF from binding to the receptor (Patel et al., 2010), and this is likely to be a further impediment to angiogenesis in the cerebral cortex in Alzheimer’s disease.

Cerebral blood flow is influenced by alterations in vascular contractility. In mice transgenic for mutant human APP, the increase in amyloid-β peptides reduced cerebral blood flow through enhanced vasoconstriction, and impaired both functional hyperaemia and cerebral autoregulation (Niwa et al., 2001, 2002a, b). We showed previously that the level of the vasoconstrictor EDN1 is increased in the cerebral cortex in Alzheimer’s disease brains (Palmer et al., 2012) in keeping with the increased synthesis of ECE1 (Palmer et al., 2010, 2013) and activity of ECE2 (Palmer et al., 2009), and also demonstrated increased ACE level and activity, an indicator of production of the vasoconstrictor angiotensin II (Miners et al., 2008). As ECE1, ECE2 and ACE are upregulated by amyloid-β peptides in vitro (Miners et al., 2009; Palmer et al., 2009, 2013), it seems probable that the elevations in EDN1 and ACE in the cerebral cortex also occur at least partly in response to the accumulation of amyloid-β peptides in vivo, although other factors may also be involved. Similar elevations in EDN1 and ACE do not occur in the white matter in Alzheimer’s disease (Barker et al., 2014), or in the cerebral cortex in dementia with Lewy bodies (Miners et al., 2014). Experimental observations raise the possibility of a vicious cycle between amyloid-β production and vasoconstriction of cortical blood vessels in Alzheimer’s disease. Amyloid-β production is increased by cerebral ischaemia in animal models (Popa-Wagner et al., 1998; Nihashi et al., 2001; Qi et al., 2007) and by oxygen-glucose deprivation of neurons in vitro (Sun et al., 2006; Zhang et al., 2007; Guglielmotto et al., 2009; Li et al., 2009), suggesting a bi-directional link between hypoperfusion and amyloid-β accumulation.

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Supplementary material

Supplementary material is available at Brain online.

References

Post-mortem assessment of hypoperfusion of cerebral cortex

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