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Key Words: hypoxia ▪ magnetic resonance imaging ▪ pathology ▪ white matter

White Matter Lesions in an Unselected Cohort of the Elderly
Molecular Pathology Suggests Origin From Chronic Hypoperfusion Injury

Malee S. Fernando, PhD; Julie E. Simpson, PhD; Fiona Matthews, PhD; Carol Brayne, MD; Claire E. Lewis, PhD; Robert Barber, MD; Raj N. Kalaria, PhD; Gill Forster, BSc; Filomena Esteves, PhD; Stephen B. Wharton, MB; Pamela J. Shaw, MD; John T. O’Brien, MD; Paul G. Ince, MD; on behalf of the ‡MRC Cognitive Function and Ageing Neuropathology Study Group

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"Incidental" white matter (WM) lesions in the aging brain appear as hypodensities on computed tomography or as hyperintensities in T2-weighted and proton density MRI sequences, which are absent from T1-weighted images.1 WM lesions (WMLs) increase in frequency with age and are associated with lower cognitive performance.2,3 They resemble the severe lesions affecting the cerebral WM in Binswanger disease and are seen in Alzheimer disease (AD). Severity of WMLs ranges from 1 small isolated foci to extensive confluent areas. WMLs may not be detected in autopsy brain tissue from examination of fresh or fixed brain slices and are not systematically included in diagnostic pathology protocols for dementia.4

Histologic studies in Binswanger disease and AD show WM changes of myelin attenuation, axonal loss, oligodendrocyte loss, astrocytic gliosis, and arteriolar sclerosis,5 interpreted as evidence for an ischemic origin. In AD, other possibilities arise, including axonal depletion from Wallerian changes attributable to cortical neuronal attrition, or toxic effects of soluble amyloid on vascular permeability. WMLs in AD patients have been described as “incomplete infarction,” although this concept is not clearly defined.5–8 Congoophilic amyloid angiopathy (CAA) is related to the presence of WMLs.5,10

The pathogenesis of “incidental” WMLs in elderly subjects is incompletely understood. Various theories have been proposed:
degenerative changes of small vessels leading to chronic cerebral hypoperfusion, altered vascular permeability and blood–brain barrier dysfunction, and cerebrospinal fluid accumulation.11,12

We used MRI of fixed coronal brain slices to enable precise sampling of WMLs.13 In WM from brains donated to the population-based MRC Cognitive Function and Ageing Study (CFAS), we compared molecular markers of tissue ischemia and indices of vascular pathology in demented and nondemented individuals. The study was conducted in 2 phases. We initially assessed vascular morphology, ependymal integrity, vascular endothelial activation (intercellular adhesion molecule [ICAM]/collagen IV [Col IV] ratio), microglial activation (CD68), and molecular markers of hypoxia-inducible factors (HIFs) HIF1α and HIF2α. We found persistent HIF expression, indicating a hypoxic environment, and we therefore conducted further work to assess the expression of other markers that we (matrix metalloproteinase-7 [MMP7], neurexin B receptor [NMBR], vascular endothelial growth factor receptor 2 [VEGFR2]) and others (neuroglobin [ Ngb]) have shown to be hypoxia regulated.14,15 The findings support an ischemic contribution to the pathogenesis of these lesions, more marked in deep subcortical lesions (DSCls) compared with periventricular lesions (PVLs).

Materials and Methods

Brain Tissue Donors
Respondents in CFAS donated the brain tissue used for the present study through a process of premortem liaison counseling approved by a research ethics committee. Dementia status was derived from longitudinal prospective collection of neuropsychological test data, including the mini mental state examination16 and Automated Geriatric Examination for Computer-Assisted Taxonomy organicity with cognitive decline and physical frailty.

Magnetic Resonance Imaging
Fixed coronal brain slices, sealed in polythene, were presented for MRI in a custom-built Perspex stack. MRI was performed at 1.0 T (Siemens) using pulse sequences optimized for postmortem WMLs: T1-weighted spin echo (2500/98 ms; TR/TE excitations, where TR = repetition time and TE = echo time); proton density (2500/25 ms); and T2-weighted inversion recovery image (TR 6383 ms, inversion time of 600 ms, with a TE of 60 ms). A single MRI “cut” was obtained at the center of each brain slice using a 5-mm slice window with an in-plane resolution of 0.63×0.59 mm.

Pathological Methods

Tissue blocks, representing WMLs and nonlesional WM, were selected using the MRI images to direct sampling. Each block was stained with hematoxylin and eosin and Luxol Fast Blue. Antibodies directed against CD68, Col IV, HIF1α, HIF2α, ICAM1, MMP7, Ngb, NMBR, and VEGFR2 were used (Table 2) using conventional immunocytochemical techniques. CAA in frontal and parietal regions overlying the WMLs studied was assessed with an antibody against β4A4. Standard immunocytochemistry (ICC) methods using dianinobenzidine (DAB) as chromogen were used, with antigen retrieval as indicated in Table 2. Negative control staining omitted primary antibody, and antibody absorption experiments were undertaken when not reported previously. Positive control sections included tumors and inflammatory brain disease as appropriate. Dual labeling with immunofluorescence and conventional DAB was used to colocalize molecular markers of tissue hypoxia and markers of glial cell phenotype. Sections were first processed for molecular markers of hypoxia using the method above, followed by incubation with avidin-biotin blocking kit (Vector Laboratories). After incubation with the cell phenotype primary antibody, the sections were incubated with biotinylated anti-mouse IgG (for CD68) or anti-rabbit IgG (for glial fibrillary acidic protein) and visualized with streptavidin tetramethylrhodamine B isothiocyanate (Serotec; red). Image capture was performed using CellR software (Olympus Biosystems) and colocalization analysis using JascPaint Shop Pro 9.

Details of the approach used to define and derive quantitative and semiquantitative measures of the molecular biological and morpho-

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TABLE 1. No. of Brains and Readings Used in Each Investigation

<table>
<thead>
<tr>
<th></th>
<th>Demented</th>
<th>Not Demented</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total (CFANS version 1)</td>
<td>99</td>
<td>108</td>
</tr>
<tr>
<td>Vascular morphology</td>
<td>11 (201)</td>
<td>8 (189)</td>
</tr>
<tr>
<td>PV</td>
<td>42 (125)</td>
<td>56 (104)</td>
</tr>
<tr>
<td>DSC</td>
<td>43 (130)</td>
<td>55 (118)</td>
</tr>
<tr>
<td>ICAM/Col IV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PV</td>
<td>12 (16)</td>
<td>26 (39)</td>
</tr>
<tr>
<td>DSC</td>
<td>14 (21)</td>
<td>30 (56)</td>
</tr>
<tr>
<td>Diffuse</td>
<td>13 (16)</td>
<td>18 (22)</td>
</tr>
<tr>
<td>Microglial activation, HIF1α and HIF2α</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PV</td>
<td>2 (3)</td>
<td>12 (16)</td>
</tr>
<tr>
<td>DSC</td>
<td>4 (5)</td>
<td>10 (19)</td>
</tr>
<tr>
<td>Total (CFANS version 3.1)</td>
<td>238</td>
<td>182</td>
</tr>
<tr>
<td>Hypoxia-regulated proteins (MMP7, NMBR, VEGFR2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>3 (5)</td>
<td>9 (10)</td>
</tr>
<tr>
<td>PV</td>
<td>6 (7)</td>
<td>5 (8)</td>
</tr>
<tr>
<td>DSC</td>
<td>5 (7)</td>
<td>6 (8)</td>
</tr>
</tbody>
</table>

†Thirty-six individuals were not demented when last seen but had no information close to death. Two are included in the analysis of hypoxia-regulated proteins; they are classified in the table as nondemented but are excluded from the analysis of dementia status at death.

CFANS indicates Cognitive Function and Ageing Neuropathology Study.
The most conservative were checked using nonparametric statistics (e.g., Kruskal–Wallis). All analyses for multiple comparisons.

Vascular Morphology

Hematoxylin and eosin sections of MRI-scanned coronal slices from a subgroup of the tissue resource were examined for qualitative assessment of vascular changes in the WM.13 Thickening of WM arterioles and small arteries was frequent. Although we did not use a specific stain appearances considered to represent periventricular venous collagenosis were rare.20 Estimations of arteriolar morphology were made in 390 arteriolar profiles from 19 brains (Table 1). Tangential or incomplete vascular profiles were avoided. The regions in which measurements were made were selected to include: nondemented individuals, WMLs (109 vessels), nonlesional WM (80 vessels); and demented individuals, WMLs (107 vessels), nonlesional WM (94 vessels).

Internal diameter was not reduced in either WMLs or in the presence of dementia (P>0.2, Figure 1a). Wall thickness within WMLs (nondemented [median] 116 μm; interquartile range [IQR] 47 to 194 μm; demented 95 μm [IQR 45 to 160 μm]) was significantly increased compared with nonlesional WM (nondemented 36 μm [IQR 21 to 96]; demented 24 μm [IQR 15 to 51; P<0.001]) and was not related to dementia (P>0.2). The perivascular space was enlarged within WMLs (P<0.001) and was slightly greater in those with dementia (P=0.04). There was no difference between cases with high neocortical neurofibrillary tangle counts (Braak stages 5 and 6; 66 vessels) compared with moderate or low counts (Braak stages 1 through 4; 324 vessels).

Ependymal Morphology

In PVLs but not DSCLs, there was increased denudation of the ventricular lining compared with the ventricular lining in the absence of WMLs (P=0.004; Figure 1b). This effect is somewhat clustered within individuals and reduces in effect when multiple readings within individuals are adjusted for (P=0.04).

ICAM/Col IV Ratio

The percentage of area stained by Col IV was similar across all of the groups studied, indicating no significant variation in the density of the capillary network (Figure 1c). There was no difference in the ratio of ICAM/Col IV in PVLs compared with PVWM (P=0.17; Figure 1d). There was a highly significant increase in the ICAM/Col IV ratio for DSCLs compared with DWM (P<0.001). We also examined 32 areas of WM that were classified radiologically as “diffuse” change,13 implying subtle radiological changes and intermediate or mild myelin attenuation by histology, which showed elevation of the ICAM/Col IV ratio compared with nonlesional DSC WM (P=0.05) but no significant difference from DSCLs (P>0.2). Analysis for age and dementia showed no significant relationship with ICAM expression (Figure 1d and 1e).

Microglial Activation and HIF Expression

The expression of CD68, HIF1α, and HIF2α was estimated in DSC and PVWM using 43 samples from 20 brains with the shortest fixation times. They consisted of 13 DSCLs and 11 DWM from 14 brains and 10 PVLs and 9 PVWM from 14 brains. The cellular morphology of HIF immunoreactivity is consistent with either microglia or astrocytes so that double-labeling techniques are required to address the cell-type specificity of expression. Quantitative differences are shown in Figure 2. Significant differences between DSCLs and DWM samples were demonstrated for CD68 (Figure 2a; P=0.002), HIF1α (P=0.003), and HIF2α (P=0.005; Figure 2a). In PVLs compared with PVWM, there was no significant difference in expression of CD68 (P>0.2). HIF1α and HIF2α expression was higher but did not reach conventional signif-

Statistical Analysis

We analyzed groups defined by MRI (“lesional” or “nonlesional”), anatomical location (PVLs or DSCLs), and dementia status (demented or nondemented). Four categories of CAA (none, mild, moderate, and severe) were collapsed to a dichotomy between “none-mild” and “moderate-severe.” Data were analyzed (STATA version 8.0 STATA Corporation) including ANOVA and Pearson χ² tests for heterogeneity. ANOVA was adjusted for multiple readings within individuals. All analyses were checked using nonparametric statistics (e.g., Kruskal–Wallis). The most conservative P values have been used. In the analysis of 3 categories (control, PVLs, and DSCLs) P values have been adjusted for multiple comparisons.

Results

Vascular Morphology

Hematoxylin and eosin sections of MRI-scanned coronal slices from a subgroup of the tissue resource were examined for qualitative assessment of vascular changes in the WM.13
There were no differences between demented and nondemented respondents (all $P>0.2$). All these relationships are affected by multiple readings within individuals; however, the sample is too small to properly adjust for this effect.

**Effects of CAA on HIF Expression**

The DWM of the centrum semiovale includes a watershed between perforating arteries derived from the cerebral cortex and long penetrating branches of arteries in the base of the brain. This arrangement potentially jeopardizes WM perfu-
Figure 2. The frequency immunoreactive profiles for CD68, HIF1α, and HIF2α (a,b,c), neuroglobin (d), MMP7 (e), neuromedin B receptor (f), and VEGF receptor2 (g) in WMLs (outliers shown as circles). HIF1α and HIF2α expression compared with severity of leptomeningeal (b) and parenchymal (a) CAA. Both are significantly associated with increased HIF1α profiles in DSCL when CAA is present at moderate/severe grades (white boxes) compared with mild/none (gray boxes).
sion in the face of any diffuse arterial disease affecting the cerebral cortex. In the CFAS study, CAA is a frequent pathological finding among both demented and nondemented respondents.18 We compared the severity of HIF1α expression in WMLs and nonlesional WM with increasing severity of CAA affecting the cortex overlying the WM regions imaged. CAA was scored (Consortium to Establish a Registry of Alzheimer Disease Protocol) into none, mild, moderate, and severe. Because dementia status did not influence HIF1α expression, DSCLs from demented and nondemented respondents were pooled and compared with CAA scores, stratified into none/mild versus moderate/severe, and analyzed separately for parenchymal and meningeal involvement.

Higher CAA load, both in parenchymal and leptomeningeal vessels, was associated with elevated HIF1α expression in DSCLs compared with DWM (Figure 2b and 2c; P=0.04). This relationship was not observed to reach statistical significance for PVLs. HIF2α expression was not related to either leptomeningeal or parenchymal amyloid load (all P>0.2).

The lack of association between HIF2α and CAA may reflect the higher constitutive levels of this protein, which is less clearly linked to the hypoxic response cascade. Double-labeling ICC for the HIFs showed HIF1α expression in both astrocytes (Figure 3) and microglia. In contrast, HIF2α expression was restricted to microglia.

Expression of Hypoxia-Regulated Proteins
The immunocytochemical demonstration of HIF1α is capricious and is highly dependent on agonal factors and postmortem delay in the human brain. To strengthen the evidence for a hypoxic response within WMLs, we quantified the frequency of cellular profiles immunoreactive for 4 other candidate markers that have been shown to be upregulated by hypoxia.14,15 There was evidence that the frequency of Ngb-expressing cell profiles were different in the 3 groups (P=0.02), being increased in DSCLs compared with control (P=0.14) and a small nonsignificant decrease in PVLs compared with control (P=0.4; Figure 2d). MMP7 immunoreactive profiles varied considerably between groups (P<0.001) with increases in both DSCLs (P=0.009) and in PVLs (P<0.001; Figure 2e). Neither NMBR-expressing (P=0.35) nor VEGFR2-expressing (P=0.25) cell profiles showed variations by lesion type, although small differences may exist (Figure 2f and 2g). Double-labeling ICC (Figure 3) shows the expression of Ngb, MMP7, NMBR, and VEGFR2 in activated microglial cells. MMP7 and NMBR are also colocal-
ized within astrocytes. Many of the cases show a population of clasmatodendritic astrocytes within both PVLs and DSCLs.

Discussion

WMLs and Markers of Hypoxia

The study demonstrated that markers associated with vascular degenerative morphology, endothelial and microglial activation, and molecular markers of a hypoxic response are altered in WMLs in the aging brain. These changes would be expected to impair, or reflect impairment of, vascular perfusion. The effect of CAA in the arterial vessels that supply DWM, and the sclerotic changes present within lesional WM, would both likely reduce perfusion in the centrum semiovale. Increased capillary endothelial activation and microglial activation are relatively nonspecific findings associated with a variety of vascular, hypoxic, and inflammatory insults, among other causes. However, the persistence of HIF-1α immunoreactive cell profiles and the increased expression of molecules that are demonstrated to be upregulated by HIF are strong evidence that WM attenuation is associated with ongoing hypoxia. HIF-1α is normally controlled via ubiquitin-mediated proteolysis through rapid degradation under normoxic conditions. HIF-1α is not readily demonstrable by ICC in normally oxygenated tissues. Persistence of HIF-1α to the extent that it becomes demonstrable by ICC is regarded as strong evidence that the tissue is in a hypoxic state. The situation regarding HIF-2α is somewhat less clear because of greater constitutive expression in normoxic conditions within macrophage lineage cells. HIF-1α is especially capricious in immunocytochemical studies, and we were only able to demonstrate staining in cases with the shortest postmortem interval between death and tissue donation and processing. We therefore also looked for the expression of various molecules known to be upregulated either directly by HIF or as a response to hypoxia and show that both MMP7 and Ngb are significantly upregulated.

PVLs Versus DSCLs

The results suggest a difference between PVM and DWM. There are anatomical differences in the vascular supply of PVWM versus DWM. The lack of an association between the severity of CAA and HIF expression in PVLs may relate to the minimal perfusion contributed by cortically derived vessels in the ventricular part of the brain. It is possible that WM attenuation may differ between PVLs and DSCLs in terms of the extent of axonal loss, demyelination, and increased interstitial fluid. The PVWM may be prone to increased fluid accumulation related to the proximity of the ventricles associated with reduced integrity of the ventricular ependyma. However, some of our data for DSCLs can be interpreted in terms of increased retention of tissue fluid related to impaired perivascular fluid drainage. In the cerebral cortex, there is a significant retrograde fluid drainage pathway associated with the perivascular spaces of arteries and arterioles. The perivascular space associated with WM and basal ganglia arteries and arterioles is contiguous with this drainage system and is likely to function in a similar way. Enlargement of perivascular spaces in WMLs may represent enlargement caused by the accumulation of tissue fluid through impaired drainage.

The observed association between the thickened arterioles with enlarged perivascular spaces and the areas of lesional WM remains unexplained. Its absence from normal WM is difficult to explain in terms of a systemic disorder such as hypertension or arteriosclerosis, which ought to cause generalized arteriolar disease. The presence of arteriolar sclerosis in WMLs has been reported previously, and our findings confirm this previous work. In a cross-sectional observational study such as this, the possibility that WM changes lead to the vascular changes or vice versa cannot be ascertained.

Of the 5 hypoxia-associated markers included in this study HIF1α, HIF2α, VEGFR2, and Ngb were significantly upregulated only in DSCLs. MMP7 was significantly upregulated in DSCLs and in PVLs. We were unable to demonstrate significant upregulation of NMBR and VEGFR2, although both markers show a nonsignificant trend toward increased expression more marked in DSCLs (Figure 2f and 2g). For the first time, we demonstrated that both astrocytes (HIF1α, MMP7, and NMBR) and microglia (HIF1α, HIF2α, Ngb, MMP7, NMBR, and VEGFR2) express molecules that are involved in the adaptive and cytoprotective response to hypoxia (Figure 3). Coexpression of HIF1α in astrocytes is a novel observation. The astrocytic population in this cohort showed clasmatodendritic changes (dendritic pruning and cytoplasmic enlargement) that have been described previously in WMLs in AD and in Binswanger disease. Clasmatodendritic astrocytes in WMLs contain plasma proteins, suggesting impairment of blood–brain barrier dysfunction and phagocytosis of extracellular proteins. It is possible that observed immunolocalization of HIF1α, MMP7, and NMBR in astrocytes is also attributable to phagocytosis. Ongoing studies, including in situ hybridization, will determine which cell types synthesize these hypoxia-response proteins and whether there is any dysregulation of the downstream adaptive pathways that account for the failure of WM to adapt to hypoxia in brain aging.

This study provides direct evidence that hypoxia plays an important role in the pathogenesis of incidental WMLs in brain aging. The pathology of these incidental WMLs includes changes demonstrated previously in the WMLs associated with AD and with Binswanger disease, suggesting that they are part of a pathological continuum common to all 3 clinical scenarios.

Acknowledgments

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References

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