Microarray RNA Expression Analysis of Cerebral White Matter Lesions Reveals Changes in Multiple Functional Pathways

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Background and Purpose—White matter lesions (WML) in brain aging are linked to dementia and depression. Ischemia contributes to their pathogenesis but other mechanisms may contribute. We used RNA microarray analysis with functional pathway grouping as an unbiased approach to investigate evidence for additional pathogenetic mechanisms.

Methods—WML were identified by MRI and pathology in brains donated to the Medical Research Council Cognitive Function and Ageing Study. RNA was extracted to compare WML with nonlesional white matter samples from cases with lesions (WM[L]), and from cases with no lesions (WM[C]) using RNA microarray and pathway analysis. Functional pathways were validated for selected genes by quantitative real-time polymerase chain reaction and immunocytochemistry.

Results—We identified 8 major pathways in which multiple genes showed altered RNA transcription (immune regulation, cell cycle, apoptosis, proteolysis, ion transport, cell structure, electron transport, metabolism) among 502 genes that were differentially expressed in WML compared to WM[C]. In WM[L], 409 genes were altered involving the same pathways. Genes selected to validate this microarray data all showed the expected changes in RNA levels and immunohistochemical expression of protein.

Conclusion—WML represent areas with a complex molecular phenotype. From this and previous evidence, WML may arise through tissue ischemia but may also reflect the contribution of additional factors like blood–brain barrier dysfunction. Differential expression of genes in WM[L] compared to WM[C] indicate a “field effect” in the seemingly normal surrounding white matter. (Stroke. 2009;40:369-375.)

Key Words: brain ischemia ■ gene microarray analysis ■ gene regulation ■ MRI ■ neuropathology ■ white matter disease

Hyperintensities are common in the white matter (WM) of elderly people detected by T2-weighted MRI studies.1–5 These white matter lesions (WML) vary in severity and can be assigned as deep subcortical lesions (DSCL) or periventricular locations. They occur in individuals with or without dementia.6–7 Both periventricular lesions and DSCL have been linked to cognitive decline and late-onset depression in older people.8–10

DSCL show significant myelin loss, oligodendroglial depletion, reactive astrogliosis, and the presence of activated microglia with phagocytic morphology.11,12 The extent of axonal pathology is not well-characterized and the causes of WML are not firmly established. WML may be prominent in Alzheimer disease attributed to a combination of local WM ischemia, sometimes mediated by congophilic amyloid angiopathy, and axonal loss secondary to cortical neurodegeneration.11,12,13 Extravasation of serum proteins has also been demonstrated in the WML of Alzheimer disease,15Binswanger disease,16 and in “incidental” lesions suggesting abnormal blood–brain barrier function. In DSCL we have previously shown evidence of tissue hypoxia.17 Nonlesional WM from cases with lesions (WM[L]) showed enhanced microglial activation compared to the WM of cases with no lesions (WM[C]), which raises the possibility that WM[L] is not in a normal state, attributable to either early degenerative or adaptive changes.18 Other work19 implicates venous col-
The changes in gene expression levels demonstrated by microarray analysis from fixed tissue. We therefore used the contralateral frozen cerebral hemisphere slices as the source of lesional and normal tissue for this study. Large DSCL are frequently bilateral but often asymmetrical.33 The MRI data available on formalin-fixed brain slices were used to select contralateral frozen slices likely to contain WML. WML and normal WM were identified in the frozen tissue using cryostat histology of blocks dissected from the frozen central hemispheric WM. We have previously demonstrated3,18 that DSCL are characterized by myelin pallor and prominent globoid microglia (CD68), whereas WM[L] and WM[C] show normal myelin and contain low levels of ramified microglia (Figure 1). These techniques were used to obtain the blocks required for the study. Because periventricular lesions occur in 92% of the elderly population,3 it was necessary to include 4 cases with periventricular lesions rated as mild (Scheltens score=1)34 to obtain the WM[C] tissue. However, these cases had only small periventricular “caps” with normal WM in the centrum semiovale. The WM samples for the study was anatomically remote from these periventricular foci and showed neither of the pathological features used to characterize DSCL. This sampling strategy, together with the subsequent elimination of cases with inadequately preserved RNA, necessitated the sequential sampling of 42 cases in the cohort until the final cohort of 14 cases was achieved.

### Isolation of RNA From WM Samples

Cryostat sections stained for CD68 were used to map DSCL to guide subdissection of ~100 mg of WML for RNA extraction. WM[L] and WM[C] blocks were similarly subdissected. Total RNA was extracted according to the manufacturer’s instructions (Qiagen’s RNeasy Lipid Tissue mini kit). RNA integrity and 28S/18S ratios were determined (Agilent 2100 Bioanalyzer; Agilent Technologies) to assess RNA quality. Tissue blocks were screened sequentially until sufficient samples with adequate RNA preservation were identified. The microarray analysis and subsequent validation studies were based on 7 cases for DSCL and WM[L] and 7 cases for WM[C]. Three cases from each group, analyzed separately, were used for the microarray analysis. The remaining cases (4 DSCL and WM[L]; 4 WM[C]) were used to validate the microarray findings.

### Identification of WML and Selection of Tissue Samples

WML demonstrated by MRI of formalin-fixed, postmortem brain correlate well with histological changes,3 but it is not possible to extract RNA adequate for microarray analysis from fixed tissue. We therefore used the contralateral frozen cerebral hemisphere slices as the source of lesional and normal tissue for this study. Large DSCL are frequently bilateral but often asymmetrical.33 The MRI data available on formalin-fixed brain slices were used to select contralateral frozen slices likely to contain WML. WML and normal WM were identified in the frozen tissue using cryostat histology of blocks dissected from the frozen central hemispheric WM. We have previously demonstrated3,18 that DSCL are characterized by myelin pallor and prominent globoid microglia (CD68), whereas WM[L] and WM[C] show normal myelin and contain low levels of ramified microglia (Figure 1). These techniques were used to obtain the blocks required for the study. Because periventricular lesions occur in 92% of the elderly population,3 it was necessary to include 4 cases with periventricular lesions rated as mild (Scheltens score=1)34 to obtain the WM[C] tissue. However, these cases had only small periventricular “caps” with normal WM in the centrum semiovale. The WM samples for the study was anatomically remote from these periventricular foci and showed neither of the pathological features used to characterize DSCL. This sampling strategy, together with the subsequent elimination of cases with inadequately preserved RNA, necessitated the sequential sampling of 42 cases in the cohort until the final cohort of 14 cases was achieved.

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### Materials and Methods

#### Human Central Nervous System Tissue

The postmortem central nervous system tissue was from brains donated to the Medical Research Council Cognitive Function and Ageing Study.37 A Research Ethics Committee approved all procedures for tissue donation and use. Brains were removed at postmortal and dissected following a standard protocol.29 One cerebral hemisphere was fixed in formalin for a minimum of 4 weeks, and the other hemisphere was sliced in the coronal plane, rapidly frozen, and stored at ~80°C. Anatomically defined formalin-fixed coronal slices underwent MRI analysis as described in detail previously.3

Gender, age at death, and brain pH may affect mRNA stability after death,30 but postmortem delay does not correlate with mRNA preservation.31,32 Therefore, we selected cases for this study with similar age at death and brain pH (Table 1) sequentially from the total resource of 506 Cognitive Function and Ageing Study brain donations on the basis of MRI appearances and the quality of extracted RNA in 2 stages. First, we identified cases with WML and nonlesional WM in frozen brain tissue from the cohort of brain donors. We then selected from these cases on the basis of quality of extracted RNA. Tissue blocks were selected into 3 comparison groups: WML (DSCL), WM[L], and WM[C].

### Table 1. Age, Sex, Postmortem Delay, and Cause of Death of MRC CFAS Brain Donors

<table>
<thead>
<tr>
<th>Age, yr</th>
<th>Gender</th>
<th>PMD, d</th>
<th>pH</th>
<th>Cause of Death</th>
<th>Lesion/Control</th>
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<tr>
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<td>7.1</td>
<td>IHD</td>
<td>WM[C]</td>
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<tr>
<td>88</td>
<td>F</td>
<td>&lt;1</td>
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<td>Pneumonia</td>
<td>WM[C]</td>
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<tr>
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<tr>
<td>87</td>
<td>F</td>
<td>&lt;1</td>
<td>6.5</td>
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</tr>
<tr>
<td>84</td>
<td>F</td>
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<td>7.0</td>
<td>Stroke</td>
<td>DSCL/WM[L]</td>
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<tr>
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<td>DSCL/WM[L]</td>
</tr>
<tr>
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<td>WM[C]</td>
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<tr>
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<td>M</td>
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<td>6.1</td>
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<td>WM[C]</td>
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<tr>
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<td>F</td>
<td>&lt;1</td>
<td>6.8</td>
<td>Diabetes</td>
<td>WM[C]</td>
</tr>
<tr>
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<td>M</td>
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<td>4.9</td>
<td>Stroke</td>
<td>WM[C]</td>
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<tr>
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<td>6.9</td>
<td>Pneumonia</td>
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<tr>
<td>91</td>
<td>F</td>
<td>&lt;1</td>
<td>6.5</td>
<td>IHD</td>
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MRC CFA indicates Medical Research Council Cognitive Function and Ageing Study; IHD, ischemic heart disease.

### Immunocytochemistry for CD68

![Figure 1. Immunocytochemistry for CD68 expression](http://stroke.aahajournals.org/content/370/2/368/F1.large.jpg)
Synthesis and Hybridization of cRNA
The target-labeled antisense RNA was prepared according to the Affymetrix protocol (all enzymes: Invitrogen); 5 µg of total RNA was annealed to an oligo-d(T) primer with a T7 polymerase binding site. First-strand cDNA was prepared using superscript II; second strand cDNA was prepared using Escherichia coli DNA ligase and polymerase I. The reaction was completed with T4 DNA polymerase and ended by adding EDTA. The amplified cDNA was cleaned using the Affymetrix clean-up module. Biotin-labeled antisense RNA was prepared using the Affymetrix Gene Chip IVT labeling kit. After clean-up of the biotin-labeled cRNA, the material was assayed (Agilent Bioanalyzer 2100) to ensure sufficient RNA of appropriate quality had been prepared. Twelve µl of labeled cRNA was fragmented and applied to HGU133 Plus 2.0 gene microarrays and hybridized for 16 hours at 45°C in a rotating oven at 60rpm. Washing after hybridization and sample staining were performed using the Fluidics Station 400 and the Gene Chip Operating System. Gene chips were scanned using the GC3000 scanner and data were processed using Gene Chip Operating System software. Further analysis was performed using Array Assist 3 software (Iobion).

Validation of Microarray Data 1: qPCR and Immunostaining for Their Protein Products
Candidate genes within 5 functional pathways showing significantly altered expression in multiple genes by microarray analysis were validated by qPCR. We selected these genes based on a combination of a high fold-change in the expression level and a central role for the gene in the pathway of interest. RNA was extracted from 4 additional DSCL with matched WM[L] and 4 additional WM[C] cases as described (Table 1). Total RNA (~1 µg) was incubated with oligo-d(T) at 65°C for 5 minutes and reverse-transcribed at 42°C for 50 minutes in a reaction mix containing superscript II. The primers were annealed to an oligo-d(T) primer with a T7 polymerase binding site. The reaction was completed with T4 DNA polymerase and ended by adding EDTA. The amplified cDNA was cleaned using cDNA reverse-transcribed from universal human RNA. A dissociation curve determined that a single PCR product, and not a primer-dimer, was produced.

The qPCR was performed using 50 ng cDNA, 1xSYBR Green PCR mastermix (Applied Biosystems, UK), optimized concentrations of forward and reverse primers, and a total volume of 20 µL. After denaturation at 95°C for 10 minutes, the products were amplified (40 cycles at 95°C for 30 sec and 60°C for 60 sec) using an MX3000P RT-PCR System (Stratagene, UK). GAPDH was amplified on each plate to normalize expression levels of target genes between different samples using the ΔΔCt calculation (Applied Biosystems) and to assess assay reproducibility.

Immunocytochemistry was performed to evaluate the expression of the protein products of these selected genes. Paraffin or cryostat sections of WML, and normal WM, were used depending on the properties of the antibodies, and with antigen retrieval when appropriate (supplemental Table I, available online at http://stroke.ahajournals.org). For negative controls, the primary antibody was omitted or sections were incubated with isotype antibody at equal antibody concentrations.

Statistical and Microarray Analysis
The gene chip–robust multi-array average (GC-RMA) algorithm was used for univariate and principle component analyses to determine intensity distribution and eliminate sample outliers. The “Pathway Assist” program was used to assign genes to specific functional pathways. Genes were considered differentially expressed if they showed a minimum 2-fold change at P<0.05. Changes in RNA levels by qPCR were compared using an unpaired t test.

Results

Microarray Analysis
The Human Genome U133 Plus 2.0 Array comprises 1.3×10⁶ unique oligonucleotide sequences, including >47 000 transcripts and variants of 33 000 genes. Between 30.5% and 40.2% of the probe set sequences were present across all samples (mean, [range]: WM[C], 34.7% [30.5%–37.2%]; DSCL, 38.7% [37.1%–40.2%]; WM[L], 36.6% [35.9%–37.3%]).

Significant differential regulation of 502 genes was observed in DSCL compared to WM[C]: 331 upregulated (supplemental Table III) and 171 downregulated (supplemental Table IV). Similarly, in WM[L], 419 genes were differentially expressed compared to WM[C]: 293 upregulated (supplemental Table V) and 126 downregulated (supplemental Table VI), compared to WM[C]. Upregulated genes greatly outnumbered downregulated genes in brains containing WML. Of the 502 transcripts differentially expressed in DSCL vs WM[C], 98 (19.5%) were also differentially expressed in WM[L] vs WM[C] (Table V). All these shared gene changes showed identical directional and similar fold change values.

The differentially expressed genes were categorized according to known molecular functions. A proportion was associated with transcriptional control or had unknown functions: 49.0% (246/502) in DSCL and 46.8% (196/419) in WM[L]. Multiple affected genes were present within 8 major genetic pathways: immune regulation, cell cycle, apoptosis, proteolysis, ion transport, cell structure, electron transport, and metabolism.

Immune regulation genes represented 10.6% (26/246) in DSCL compared to WM[C]. Upregulated genes were involved antigen presentation, complement, lymphocyte activation, proinflammatory cytokine signaling, and phagocytosis. Proteolysis genes represented 8.1% (20/246) in DSCL compared to WM[C] including members of the matrix metalloproteinase family and proteases associated with remodeling of the extracellular matrix. Cell-cycle genes represented 8.9% (22/246), including upregulation of genes involved in cell-cycle progression and control. The transcription factor E2F1, which controls cell-cycle timing, was downregulated and associated with downregulation of genes dependent on E2F1 expression, including CDC6. A majority of transcripts associated with Na⁺, Ca²⁺, K⁺, and Cl⁻ channels (22/29) were upregulated. Transcripts associated with metabolism were also upregulated in DSCL (17/21) coding for molecules involved in glycolysis breakdown, carbohydrate metabolism, lipid metabolism, and glyceral metabolism. Whereas 4.8% (11/246) of differentially regulated genes in DSCL were related to apoptosis signaling, there was no clear predominance of proapoptotic or antiapoptotic genes. Genes associated with cell adhesion (7/246; 2.8%), structure (14/246; 5.6%), and transport (5/256; 2.0%), and the majority of genes associated the formation or regulation of cytoskeletal components (11/14), were upregulated. In contrast, all transcripts associated with vesicle trafficking and cellular transport were downregulated. Most transcripts associated with the electron transport chain were upregulated in DSCL compared to WM[C] (3.2%; 8/246).
were upregulated and code for proteins associated with regulation of cytoskeletal components, actin-binding, and junctional proteins. Another significant category of differentially expressed genes in WM[L] were associated with ion transport (26/196, 13.2%). In common with DSCL, there was differential regulation of genes in the proteolysis pathway (8.1%, 16/196), including members of the matrix metalloproteinases superfamily and the ubiquitin–proteasome pathway. These ubiquitin–proteasome pathway genes were downregulated in WM[L] compared to WM[C]. Among the transcripts involved in immunity, 5.6% (11/196) upregulated genes related to antigen presentation, lymphocyte activation, proinflammatory cytokine signaling, and phagocytosis. As in DSCL, the majority of genes associated with cell-cycle progression (5.1%; 10/196) and control were upregulated.

Transcripts associated with the electron transport chain (3.6%; 7/223) included 2 brain-specific electron transport protein cytochrome b-561 transcripts that were upregulated similar to DSCL. In contrast to DSCL, only one gene associated with apoptosis signaling was differentially expressed in WM[L].

**Validation of Microarray Data**

We selected 8 genes from 5 of the functional pathways highlighted by the microarray data to validate the quantitative RNA findings and to look for changes in protein expression in DSCL compared to WM[C]. The selected candidates from each pathway are shown in Table 2.

The qPCR analysis confirmed upregulation of human leukocyte antigen-DQ B1 (Figure 2A) and CD14 (Figure 2B) as seen in DSCL compared to WM[C] in the microarray analysis. The highest levels of CD14 were associated with WM[L]. Immunostaining for MHC class II shows MHC II+ microglia with amoeboid morphology within DSCL (Figure 3A), but only occasional positive microglia in WM[C] (Figure 3B), reflecting increased human leukocyte antigen-DQ B1 expression. The qPCR analysis confirmed increased RNA transcription of cathepsin-B in DSCL compared to WM[C] (Figure 2C) and there was increased staining of microglia and astrocytes in DSCL (Figure 3C to 3D). Cathepsin-C staining in WM[C] showed low-level expression in occasional ramified microglia (Figure 3E) compared to prominent amoeboid microglia in DSCL (Figure 3F). Glutamate α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate

![Figure 2. Validation of microarray findings by qPCR analysis. In these 6 genes selected for validation, the qPCR data parallel that from the microarray analysis. Note that the changes in CD14 (B), GR1AI (D), and CDC6 (F) are maximal in WM[L].](image-url)
(AMPA) (Figure 3I and 3J) and kainate receptor (Figure 3K and 3L) expression were significantly upregulated in both DSCL and WM[L] compared to WM[C] as confirmed by qPCR for GRIA1 (Figure 2D). Caspase-2, an apoptosis-related gene, showed increased expression in DSCL compared to WM[C] by microarray analysis confirmed by qPCR (Figure 2E). Increased expression of caspase-2 protein was demonstrated in DSCL (Figure 3G and 3H). The cell-cycle gene CDC6 was decreased in DSCL compared to WM[C] in both the microarray and qPCR studies (Figure 1F). Altered expression of CDC6 was demonstrated by immunocytochemistry.

Discussion

We present gene expression profile data of WML and nonlesional WM in the aging brain compared to WM from cases with no lesions. The majority of significantly differentially regulated transcripts in DSCL and WM[L], with known function, code for genes associated with immune function, the cell cycle, proteolysis, and ion transport. Changes were also observed in pathways associated with electron transport, metabolism, and cell structure. We validated these microarray data using qPCR and immunocytochemistry. RNA microarray expression studies have inherent limitations in terms of their ability to elucidate the initiating events or tissue insults that result in a pathological phenotype. However, these limitations are balanced by the advantages of an unbiased approach and the genome-wide evaluation of transcription. When combined with a pathway analysis procedure, RNA expression microarrays are able to demonstrate multiple candidate pathways that contribute to the development of pathology, any of which may offer real prospects for therapeutic development against definable molecular targets. Although many genes represented on the microarray chips are of unknown function, reducing the precision of the pathway analysis, the data can be reanalyzed in the future as more of these genes are characterized.

Nonlesional deep subcortical WM from brains that contain WML (WM[L]) is not abnormal by MRI. However, WM[L] contains significantly higher levels of activated microglia (MHC II expression) compared to WM from controls with absent WML (WM[C]).18 The significance of these “extraoral” changes is unknown and it is unclear if microglial activation is a protective response or if it contributes to progression toward WML. DSCL contained 502 differentially regulated genes compared to WM[C], but there were also 419 genes differentially regulated in WM[L], providing further evidence of active processes in WM outside of the DSCL. Both DSCL and WM[L] show similar RNA transcription expression patterns, so the majority of differentially regulated genes of known function are in the same pathways. Both these new gene expression data and our previous molecular pathology study18 suggest that WML arise in the context of more widespread changes in the central WM.

Approximately 11% of the differentially expressed genes in both WM[L] and DSCL were associated with ion transport. Most transcripts were upregulated in both groups including genes involved in the formation or function of Na+/K+/Ca2+ ion channels. Myelinated axons are highly susceptible to changes in ionic gradients, and changes in ion channel function, particularly Na+ channels, can result in axonal degeneration.36 There are remarkably little data on axonal loss in WML, and it is unclear if these alterations in ion channel gene expression are pathogenic or compensatory. Increased expression of genes associated with the electron transport chain was detected in both DSCL and WM[L]. Mitochondrial changes in WML might be triggered by tonic imbalance, resulting in intracellular Ca2+ accumulation.37,38 There is also evidence that modulation of ion channel proteins and receptors, including the AMPA/kainate receptor, can regulate the proliferation and differentiation of cells of oligodendrocyte lineage.39 Our finding that the glutamate receptor AMPA1 transcript was highly upregulated in both DSCL and WM[L] may indicate that oligodendrocytes are primed to attempt remyelination in the aging brain, but it has also been observed that elevated expression of glutamate receptors in aging WM contributes to its increased vulnerability to ischemia.39

Immune-related genes were differentially expressed in both WML and WM[L]. Genes involved in antigen presentation, eg, MHC II, were upregulated in DSCL consistent with previous findings that microglial activation is a major feature of WML and helps differentiate WM[L] from WM[C]
in pathological studies.18 Upregulated expression of phagocytic markers, proinflammatory cytokines, and lymphocyte activation transcripts provide further evidence of an immune response. Whether the sustained expression of immune-related proteins promotes or counteracts the pathogenesis of WML formation is not resolved and it is not clear what physiological stimuli are responsible. In addition to contributing to an immune response, proinflammatory cytokines such as the interferon and tumor necrosis factor families induce genes that regulate apoptosis,40 cell signaling, and metabolism.41 In DSCL, apoptosis-related transcripts accounted for 4% of the differentially expressed genes, whereas only 1 was significantly altered in WM[L]. It is likely that apoptosis plays a role in the pathogenesis of WML. Triggering of apoptosis may be a key step in lesion formation and account for the lack of upregulation in WM[L] associated with preserved oligodendroglia.

Changes in genes regulating proteolysis are a feature of both DSCL and WM[L]. DSCL show increased expression of cathepsin-B, expressed at high levels by amoeboid microglia and astrocytes,42 and cathepsin-C. These lysosomal proteases degrade proteins including myelin basic protein,43 suggesting a role in myelin loss in WML. Cathepsin-B contributes to antigen presentation through human leukocyte antigen-DR cleavage to make the antigen site accessible.44 Matrix metalloproteinase family proteins were also upregulated in DSCL and WM[L], and may contribute to myelin degradation, breakdown of the extracellular matrix, and blood–brain barrier disruption.45

Upregulation of genes involved in cellular adhesion and cytoskeletal homeostasis in DSCL and WM[L] may reflect recruitment and differentiation of glial cells. A number of cell-cycle and cell-cycle control genes were upregulated and, although we previously reported that protein levels of minichromosome maintenance protein 2 and proliferating cell nuclear antigen by immunocytochemistry are not significantly different between DSCL, WM[L], and WM[C],12 this was not based on RNA transcripts.

We previously showed that DSCL are associated with hypoxia, resulting in the stabilized expression of hypoxia-related proteins, including hypoxia-inducible factor-1α and hypoxia-inducible factor-2α. We also showed upregulation of genes induced by hypoxia, including matrix metalloproteinase-7.17 In the microarray data, the expression of RNA for hypoxia-inducible factor, matrix metalloproteinase-7, neurogliolin, and vascular endothelial growth factor were increased but did not reach the arbitrary significance levels set. The microarray approach is not able to discriminate the underlying, fundamental, pathogenic insult from secondary changes in cellular function and pathophysiology. Our data are therefore compatible with a primary ischemic origin for WML, but increase the range of pathways in which target identification for therapeutic intervention might be considered.

In summary, we identified a number of genes that may be relevant to the pathogenesis of WML associated with aging. WML show activation of multiple cellular pathways central to injury responses and inflammation. They appear to arise in a background of WM showing active cellular and molecular processes but that are distinct from those demonstrated in WML. The majority of these genes and functional pathways has not been examined with respect to the pathogenesis of WML and are revealed as targets for future studies.

Disclosures

None.

References


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