2.2

Defective glia maturation in vanishing white matter

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Abstract

Vanishing white matter (VWM) disease is a genetic leukoencephalopathy linked to mutations in the eukaryotic translation initiation factor 2B. It is a disease of infants, children, and adults who experience a slowly progressive neurologic deterioration with episodes of rapid clinical worsening triggered by stress and eventually leading to death. Characteristic neuropathologic findings include cystic degeneration of the white matter with scarce reactive gliosis, dysmorphic astrocytes, and paucity of myelin despite an increase in oligodendrocytic density. To assess whether a defective maturation of macroglia may be responsible for the feeble gliosis and lack of myelin, we investigated the maturation status of astrocytes and oligodendrocytes in the brains of 8 VWM patients, 4 patients with other white matter disorders and 6 age-matched controls with a combination of immunocytochemistry, histochemistry, scratch-wound assays, Western blot, and quantitative polymerase chain reaction. We observed increased proliferation and a defect in the maturation of VWM astrocytes. They show an anomalous composition of their intermediate filament network with predominance of the δ-isoform of the glial fibrillary acidic protein and an increase in the heat shock protein αB-crystallin, supporting the possibility that a deficiency in astrocyte function may contribute to the loss of white matter in VWM. We also demonstrated a significant increase in numbers of premyelinating oligodendrocyte progenitors in VWM, which may explain the coexistence of oligodendrocytosis and myelin paucity in the patients’ white matter.
Introduction

Leukoencephalopathy with vanishing white matter (VWM; OMIM number 603896) (van der Knaap et al., 1997), also referred to as childhood ataxia with diffuse central nervous system (CNS) hypomyelination (Schiffmann et al., 1994) and myelinopathia centralis diffusa (Brück et al., 2001), is one of the most prevalent inherited childhood white matter disorders (van der Knaap et al., 2006). Vanishing white matter disease is caused by mutations in any of 5 genes that encode the eukaryotic translation initiation factor 2B (Leegwater et al., 2001; van der Knaap et al., 2002), a ubiquitously expressed heteropentamer involved in the regulation of protein synthesis. Clinically, VWM is characterized by progressive cerebellar ataxia with spasticity and cognitive decline. Vanishing white matter is typified by episodes of major neurologic deterioration triggered by mild stresses as fever and minor trauma, which may lead to unexplained coma and death (van der Knaap et al., 2006).

The radiographic and pathologic findings in VWM are distinctive (Bugiani et al., 2010). The loss of white matter documented on MRI correlates with increasing rarefaction and cystic degeneration of the tissue, which is eventually replaced by fluid. Macroglia are selectively involved. In and around the areas of cavitation, the degree of reactive gliosis is disproportionately low relative to the extent of white matter damage, whereas those reactive astrocytes that are found exhibit overtly aberrant morphology (van der Knaap et al., 2006; Bugiani et al., 2010; Rodriguez et al., 1999). In less involved areas of relative preservation, lack of myelin is accompanied by increased density of oligodendrocytes identified by their morphologic and immunohistochemical properties (Rodriguez et al., 1999; van Haren et al., 2004).

This study was designed to investigate the nature of glial pathology in VWM, in particular the role of astrocytes in the cystic degeneration of the white matter. In addition, we sought to understand the paradox of lack of myelin in the face of increased oligodendrocytic density. In addressing both issues, we asked whether defective glial maturation might represent a critical causal determinant of VWM.

Materials and methods

Patients

Brain tissue from 8 genetically proven VWM patients was collected at the VU University Medical Center in Amsterdam, the Netherlands, or obtained from the
Brain and Tissue Bank for Developmental Disorders at the University of Maryland, Baltimore, MD. The nonneurologic controls included 5 subjects without significant or confounding neuropathologic findings at autopsy. Additional control tissue was obtained from an adult who underwent therapeutic surgical resection for refractory epilepsy. In addition, 4 disease controls were used: 1 patient with juvenile-onset X-linked adrenoleukodystrophy (X-ALD), 1 child with infantile Krabbe disease (globoid cell leukodystrophy, GLD), 1 patient with adolescent-onset Alexander disease (AXD), and 1 adult with multiple sclerosis (MS; table 1). The study was approved by the institutional review board. Informed consent was obtained in all cases. Tissue from patients VWM295 and VWM80 has been used in previous studies (van der Voorn et al., 2005; Dietrich et al., 2005; van Kollenburg et al., 2006).

The study focused on frontal lobe white matter, and brain specimens were selected from relatively preserved subcortical areas adjacent to cavitory foci. Corresponding areas were selected from controls. Because of the different stages of disease that influenced the amount of white matter obtainable and different abundance of control tissue, not all VWM patients and controls could be used for each experiment (table 1).

**Immunohistochemical staining**

Tissue from 5 VWM patients, 3 nonneurologic controls and 4 neurologic controls was snap-frozen and conserved at -80°C. Twenty-micrometer-thick sections were mounted on glass slides, fixed in 1% or 4% paraformaldehyde, subsequently permeabilized with 0.1% saponin, blocked in 5% normal donkey serum, and incubated with primary antibodies for 24 to 72 hours at 4°C. In addition, tissue from Patient VWM367 was formalin-fixed and paraffin-embedded, and sections were deparaffinized and stained according to standard protocols. Immunohistochemistry was performed using the antibodies listed in Table 2. Negative controls, by omitting the primary antibody, were included in each experiment. After staining with secondary antibodies (Alexa 488-, 568-, and 594-tagged antimouse, antirabbit, and antichicken IgG and antimouse IgM; 1:400; Molecular Probes; Invitrogen, Breda, the Netherlands), sections were counterstained with 4′-6-Diamidino-2-phenylindole (DAPI) (10 ng/mL; Molecular Probes; Invitrogen, Breda, the Netherlands) and photographed using a Leica DM6000B microscope (Leica Microsystems BV, Rijswijk, the Netherlands) or an Olympus FluoView 300 confocal microscope (Olympus Inc., Tokyo, Japan). Immunopositivity on paraffin-embedded material
### Table 1. Demographic data and usage of patient material

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age</th>
<th>Clinical Diagnosis</th>
<th>Usage</th>
</tr>
</thead>
<tbody>
<tr>
<td>VWM295</td>
<td>4.5 mo</td>
<td>Severe VWM; α, Gly200Val/Pro291Ser*</td>
<td>IHC, WB, qPCR</td>
</tr>
<tr>
<td>VWM251</td>
<td>4 yr</td>
<td>Severe VWM; ε, Thr91Ala/Val437Met*</td>
<td>Primary astrocytic culture, ICC</td>
</tr>
<tr>
<td>VWM576</td>
<td>6 yr</td>
<td>Severe VWM; ε, Thr91Ala/Val437Met*</td>
<td>Primary oligodendrocytic and astrocytic culture, ICC</td>
</tr>
<tr>
<td>VWM367</td>
<td>10 yr</td>
<td>Classic VWM; ε, Arg113His/Ala403Val*</td>
<td>IHC, qPCR</td>
</tr>
<tr>
<td>VWM80</td>
<td>12 yr</td>
<td>Classic VWM; ε, Thr91Ala/Trp628Arg*</td>
<td>IHC, WB, qPCR</td>
</tr>
<tr>
<td>VWM99</td>
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<td>Classic VWM; ε, Thr91Ala/Trp628Arg*</td>
<td>IHC</td>
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<td>VWM3</td>
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<td>Mild VWM; ε, Thr91Ala/Thr91Ala*</td>
<td>IHC, WB, qPCR, primary astrocytic culture, ICC</td>
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<tr>
<td>VWM44</td>
<td>36 yr</td>
<td>Mild VWM; ε, Arg113His/Arg133His*</td>
<td>IHC, primary astrocytic culture</td>
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<td>29 w GA</td>
<td>Nonneurologic control (intrauterine sepsis)</td>
<td>Primary astrocytic culture, ICC</td>
</tr>
<tr>
<td>Control 2</td>
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<td>Nonneurologic control (multiple traumatic injuries)</td>
<td>IHC, WB, qPCR</td>
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<td>Control 3</td>
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<td>Nonneurologic control (multiple traumatic injuries)</td>
<td>IHC, WB, qPCR</td>
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<td>Control 4</td>
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<td>Nonneurologic control (brachio-oto-renal syndrome)</td>
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<td>Control 6</td>
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<td>IHC, qPCR</td>
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<td>Krabbe disease</td>
<td>IHC</td>
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<tr>
<td>Control 8</td>
<td>12 yr</td>
<td>X-linked adrenoleukodystrophy</td>
<td>IHC</td>
</tr>
<tr>
<td>Control 9</td>
<td>29 yr</td>
<td>Alexander disease</td>
<td>IHC</td>
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<tr>
<td>Control 10</td>
<td>66 yr</td>
<td>Multiple sclerosis</td>
<td>IHC</td>
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mo, months; yr, year(s); w GA, weeks gestational age. *The mutant eIF2B subunit and amino acid changes are indicated. GA indicates gestational age; ICC, immunocytochemistry; IHC, immunohistochemistry; qPCR, quantitative PCR analysis; VWM, vanishing white matter disease; WB, Western blot analysis.
was detected with 3,3'-diaminobenzidine as chromogen.

Quantification and statistical analysis

The total numbers of labeled and unlabeled cells were counted in at least 10 standardized fields using a 10× objective lens. For each stained section, immunopositive cells were counted individually and expressed as a percentage of the total number of DAPI-positive cells to avoid bias due to a different cell density. The results of these counts were expressed as means ± SDs and compared using an unpaired Student t test. The Prism4 software (Graph Pad Software, Inc, San Diego, CA) was used for these analyses.

SDS-PAGE and Western blotting

Cell lysates of white and gray brain matter from 3 VWM patients and 2 controls were obtained, as previously described (van der Voorn et al., 2005). In brief, approximately 1 mg of material was homogenized in NP40 lysis buffer containing a protease inhibitor cocktail (Roche, Indianapolis, IN) with 1 mmol/L dithiothreitol. The supernatants were run on 4% to 12% SDS-polyacrylamide Precast gels (Invitrogen) and transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon P; Millipore, Billerica, MA). The blots were incubated with antibodies against glial fibrillary acidic protein δ (GFAPδ) and the endogenous protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to control equal loading. Proteins were visualized using alkaline phosphatase-coupled secondary antibodies and ECF Western blot detection reagent (Amersham, Piscataway, NJ).

RNA isolation, reverse transcription, and real-time quantitative polymerase chain reaction

White matter samples from 4 VWM patients and 4 non-neurologic controls were used for real-time quantitative polymerase chain reaction (qPCR) analysis of GFAP isoforms. RNA was extracted from frozen tissue with TRizol (Invitrogen, La Jolla, CA) and purified using RNaseasy (Qiagen, Chatsworth, CA) according to manufacturers’ specifications. Subsequent reverse transcription to complementary DNA was performed with SuperScript III reverse transcriptase (Invitrogen) and qPCR by using a LightCycler 480 II (Roche, Woerden, the Netherlands). Transcript-specific primers were generated with Primer Express software (Applied
Biosystems, Foster City, CA) and designed to overlap exon-exon boundaries to prevent genomic DNA amplification (Table 2). The PCR was carried out using a volume of 10 µl containing SYBR green PCR mix, 3.0 µmol/L primers, and 0.03 µg of complementary DNA. The relative abundance of transcript expression was calculated using the cycle of threshold value and normalized to the endogenous controls GAPDH, hypoxanthine-guanine phosphoribosyl transferase, cyclophilin B, and ribosomal protein, large, P0.

**Primary astrocyte cultures and scratch-wound assay**

Primary astrocytes from 4 VWM patients and 3 non-neurologic controls were grown in DMEM/F12 + GlutaMAX (Invitrogen) + 10% fetal calf serum (HyClone, Thermo Scientific, Etten-leur, the Netherlands) + 1 mmol/L sodium pyruvate (Invitrogen) + 1× G5 supplement (Invitrogen) to a maximum of 6 passages. Cells were then plated on poly-l-lysine (P4707; Sigma-Aldrich, Zwijndrecht, the Netherlands)-coated 12-well plates with clear bottoms (Corning Costar 3712, VWR, Amsterdam, the Netherlands) at a density of 1 × 106 cells/well in a volume of 1 ml and incubated overnight (37°C, 5% CO2). Scratching was performed 24 hours later using a custom-made sterile aluminum scratch mold (cross with leg length 10 mm, gap diameter of 1 mm) and a sterile p10 filter tip to ensure standardized scratches. With the device in the well, the pipette tip was placed in the top-corner and pulled toward the user. Plates were fixed with 4% paraformaldehyde immediately or after a 24-, 48-, or 72-hour incubation. After nuclear counterstain with DAPI (0.1 µg/ml, D9542; Sigma-Aldrich), images were acquired in an automated fashion using a Cellomics ArrayScan VTI HCS Reader (Cellomics, Inc., Pittsburgh, PA). Cell locations were determined using the accompanying software based on their nuclear staining. The resulting 49 images per well (and corresponding data sets of cell locations) were subsequently integrated using an in-house developed tool to determine the numbers of cells inside or outside the scratch area. The same scratch area definition was used for all wells.

**Magnetic sorting of oligodendrocyte progenitor cells**

Frontal white matter from Patient VWM367 was separated from the gray matter, collected in Hank’s Balanced Salt Solution (HBSS) with magnesium and calcium (Invitrogen), and then dissected and dissociated to a single-cell suspension using
papain as described (Roy et al., 1999). The cells were suspended at $4 \times 10^6$ cells/mL in DMEM/F12/N1 + GlutaMAX (Invitrogen) with basic fibroblasts growth factor (bFGF) (20 ng/mL; Invitrogen) in 100-mm TC-treated culture dishes (Corning Costar) and incubated overnight (37°C, 5% CO2).

The day after dissociation, the cells were washed twice in HBSS without magnesium and calcium (Invitrogen), incubated with magnetically labeled anti-A2B5 beads (Milteny Biotec, Utrecht, the Netherlands), and sorted using positive selection columns according to the manufacturer's protocol. To differentiate A2B5-positive glia progenitors into oligodendrocytes, cells were plated at 50,000 cells/well in double coated poly-L-lysine (P4707; Sigma-Aldrich) + laminin (10 µg/mL, L6274; Sigma-Aldrich) 8-well Labtek chamber slides (NUNC, VWR), in 400 µL of DMEM/F12/N1 + GlutaMAX (Invitrogen) with neurotrophin-3 (2 ng/mL, N1905; Sigma-Aldrich), and thyroid hormone T3 (2 ng/mL, T5516; Sigma-Aldrich) for 72 hours. Cells were then fixed with 4% paraformaldehyde before immunocytochemistry (table 2).

**Results**

Hematoxylin and eosin-stained tissue sections of the VWM patients showed white matter rarefaction with myelin paucity and increased cellular density. The U-fibers were relatively preserved (fig. 1A). Many cells in the VWM white matter exhibited oligodendroglial morphologies. Around areas of white matter vacuolation, astrocytes with coarse, blunt processes were also identified (fig. 1B,C). Remarkably, despite the overtly aberrant appearance of white matter astroglia, their counterparts in adjacent gray matter regions appeared normal (fig. 1D,E). Histopathology of the 4 neurologic controls was consistent with their respective diagnoses (Powers, 2004). No significant abnormalities were found in the non-neurologic controls.

**Astrocyte Pathology in Vanishing White Matter**

*Astrocytes proliferate in VWM in vivo and in vitro*

To investigate the meager reactive gliosis in VWM, we first determined whether astrocytes actively proliferate in VWM lesions. Compared with controls, there was a significantly increased proportion of GFAP-positive astrocytes in the tissue from VWM patients that stained positively for the proliferation marker MIB-1/Ki-67 (fig.
<table>
<thead>
<tr>
<th>Antibody</th>
<th>Marker</th>
<th>Dilution</th>
<th>Vendor</th>
<th>Catalog no.</th>
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<td>Ki67 (Mib-1)</td>
<td>Cell proliferation</td>
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<td>Neomarkers</td>
<td>cl. SP6, RM-9106-S1</td>
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<td>GFAP</td>
<td>Astrocytes</td>
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<td>Millipore</td>
<td>AB5541</td>
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<tr>
<td>S100β</td>
<td>Astrocytes</td>
<td>1:200</td>
<td>Sigma</td>
<td>S2532</td>
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<tr>
<td>Vimentin</td>
<td>Astrocytes</td>
<td>1:2000</td>
<td>cl. V9 (a)</td>
<td></td>
</tr>
<tr>
<td>Nestin</td>
<td>Astrocytes</td>
<td>1:200</td>
<td>Millipore</td>
<td>MAB5623</td>
</tr>
<tr>
<td>GFAPδ</td>
<td>Astrocytes</td>
<td>1:500</td>
<td>(b)</td>
<td></td>
</tr>
<tr>
<td>aB-crystallin</td>
<td>Astrocytes</td>
<td>1:1000</td>
<td>Novostra</td>
<td>ABCRYS-512-U</td>
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<tr>
<td>CD44 (Hermes3)</td>
<td>Astrocyte progenitors</td>
<td>1:1000</td>
<td>(c)</td>
<td></td>
</tr>
<tr>
<td>Olig2</td>
<td>OPC/Oligodendrocytes</td>
<td>1:400</td>
<td>Abcam</td>
<td>ab33427</td>
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<td>PDGFγ (CD140a)</td>
<td>OPC</td>
<td>1:100</td>
<td>BD Pharmigen</td>
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<tr>
<td>NG2</td>
<td>OPC</td>
<td>1:100</td>
<td>Millipore</td>
<td>MAB2029 (cl. 9.2.27)</td>
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<td>Millipore</td>
<td>AB142</td>
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<tr>
<td>GalC (O1)</td>
<td>Oligodendrocytes</td>
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<tr>
<td>GAPDH</td>
<td>Constitutively expressed</td>
<td>1:500</td>
<td>Abcam</td>
<td>Ab9485</td>
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</table>

**q-PCR primer (5'-3')**

| GFAPδ, forward     | CCTACAGGAAGCTGCTAGAG |
| GFAPδ, reverse     | GCGTTCATTTACAATCTGGT |
| Total GFAP, forward| AGAAGCTCCAGGATGAAACC |
| Total GFAP, reverse| TTCATCTGCTTCTGTCTATAGG |
| GFAPα, forward     | AGAGGTCTAAGAGGAGTCCA |
| GFAPα, reverse     | CAACTATCCTGCTCTGTCTC |
| GAPDH, forward     | CTCTCTGCTCCTCTGTCTCGAC |
| GAPDH, reverse     | TGAGCGATGCGGTCGCGCT |
| HPRT, forward      | ATGGGAGGCCATACACATGGT |
| HPRT, reverse      | ATGTAATGCCAGGAGTCGCAA |
| RPLP0, forward     | GTGATGTCAGCTGATCAAGCT |
| RPLP0, reverse     | GATGACCAGCCAAAGAGAA |
| CypB, forward      | AAGGACTTCATGATCCAGGG |
| CypB, reverse      | TGAAGTTCTCATCGGGAGA |

(a) Antibody manufactured at the Department of Pathology, VU University Medical Center, Amsterdam, the Netherlands. (b) Antibody manufactured at the Netherlands Institute for Neurosciences, Amsterdam, the Netherlands (Middeldorp et al., 2010). (c) Antibody kind gift from E. Aronica, University of Amsterdam, the Netherlands.
Figure 1. General neuropathologic features of vanishing white matter disease. (A) Low-power magnification hematoxylin and eosin staining of the white matter (right side of field) from the frontal lobe of Patient VWM367 shows tissue rarefaction and increased cellular density. The U-fibers appear relatively preserved; the overlying cortex is normal. (B, C) At higher magnification, white matter astrocytes display abnormal morphology with coarse blunt processes. (D, E) The cortical architecture and morphology of cortical astrocytes are normal. (A, B, D) Hematoxylin and eosin; (C, E) immunohistochemistry for glial fibrillary acidic protein. Original magnifications: (A) 25x; (B-E) 200x.
2A). Some of these Ki-67-positive/GFAP-positive cells displayed incomplete nuclear separation or frank karyokinesis without cytokinesis (fig. 2B).

Consistent with these observations, scratch-wound assays performed on primary astrocyte cultures from 4 VWM patients and 3 non-neurologic controls showed that the slope of the cell growth inside the scratch area was markedly steeper in the cultures from the VWM patients, indicating a faster cell growth rate (fig. 2C,D).

**Astrocytes in VWM remain immature and express galactocerebroside and olig2**

There was consistent and robust coexpression of vimentin and nestin in GFAP-positive cells from the VWM patients, suggesting immaturity (fig. 2E). Consistent with this, no nuclear S100β immunolabeling was detected (fig. 2F). By contrast, virtually all astrocytes in non-neurologic disease control samples were nestin-negative and S100β-positive (fig. 2G).

Aberrant coexpression of GFAP together with the oligodendrocyte-specific marker galactocerebroside (GalC) was previously described in vitro (Dietrich et al., 2005). This was interpreted as indicating a mixed lineage or immature cell type. GalC immunostaining on white matter sections from all VWM and controls samples and 2 anti-GalC antibodies from different manufacturers yielded identical results. Apparent astrocytes in the VWM patient white matter expressed GalC in a diffuse fashion in the cytoplasm of the cell body and processes (fig. 3A-C). Similar, although less intense, GalC expression was found in the white matter of the GLD patient, both in GFAP-positive astrocytes and in giant multinucleated GFAP-negative globoid cells. Rare and faintly GalC-positive astrocytes were found in the MS plaque and within an active demyelinating area in the X-ALD case. However, in both latter conditions, GalC-labeled astrocytes displayed punctate rather than diffuse immunoreactivity. No GalC expression was found in the AXD astrocytes or in non-neurologic disease controls.

Some VWM astrocytes also coexpressed the oligodendrocyte transcription factor olig2. In these cells, olig2 immunoreactivity was in the astrocyte cytoplasm (fig. 3D). In contrast, no olig2-positive astrocytes were found in sections from the neurologic and non-neurologic disease controls. This finding was also documented in glia progenitor cells sorted from the white matter of Patient VWM576. When cultured in a medium specific for oligodendrocytes, cells expressed olig2 in the nuclei, consistent with their oligodendrocytic differentiation. However, cells were also found with both nuclear and cytoplasmic olig2.
Figure 2. Vanishing white matter disease (VWM) astrocytes proliferate actively but do not reach full maturity. (A) There are significantly higher numbers of Ki-67-positive astrocytes in the white matter of VWM patients than in controls (n = 5 patients, 3 controls; p<0.0001; bars=SD). (B) Double stain for glial fibrillary acidic protein (GFAP) and Ki-67 shows cells with incomplete separation of the nucleus and
immunoreactivity and bipolar morphology, suggesting that there was a transition from oligodendrocyte-committed progenitor cells to astrocyte progenitor cells. Consistent with this, these cells expressed GFAP (data not shown) and the astrocyte progenitor marker CD44 (fig. 3E).

**Dysmorphic astrocytes in VWM overexpress both GFAPδ and αB-crystallin**

To gain insight into the morphology of VWM astrocytes, tissue sections were immunostained for GFAPδ, a protein isoform of GFAP. Astrocytes throughout VWM white matter were uniformly GFAPδ-positive, showing intense immunoreactivity in the cytoplasm that extended far into the processes (fig. 4A). No GFAPδ-positive astrocytes were detected in the white matter from normal controls. Western blotting of cell extracts of white matter from 3 VWM patients and 2 age-matched controls confirmed the immunohistochemical data, that is, GFAPδ was clearly detectable in the white matter of the VWM patients but not in the controls (fig. 4B). To investigate GFAPδ upregulation at the messenger RNA (mRNA) level, qPCR was performed on corresponding white matter areas from 4 VWM patients and 4 nonneurologic disease controls. Although GFAPδ transcripts were weakly expressed in the control tissues (even in the absence of readily detectable protein), these GFAPδ transcripts were, on average, 5 times higher in VWM (fig. 4C). Importantly, we found no significant difference between VWM and controls with respect to the message levels of the predominant isoform GFAPα (fig. 4C). To assess the specificity of this finding, immunostaining was extended to the 4

**Figure 2 (cont.).** abnormal morphology with blunt processes (Patient VWM3). (C) Scratch-wound assays of Control 5 (left panel) and Patient VWM44 (right panel) show increased proliferation of primary cultured astrocytes in the scratch 48 hours after administration of a mechanical stress. Nuclei of the proliferating cells inside the scratch area are blue. Nuclei of the unstressed cells outside this area are red; their net number is not changed versus controls. (D) The slope of proliferation in the scratch area; the individual net values are indicated as dots (n = 4 patients, 3 controls; p=0.06; the lack of significance is due to the large variance of cell growth among the different VWM patients versus controls). (E, F) GFAP-positive astrocytes from VWM patients express the intermediate filament nestin (Patient VWM99) (E) and are S100β-negative (F) (Patient VWM3), suggesting that they are immature. (G) Astrocytes from controls show normal morphology with fine arborizations and both nuclear and cytoplasmic S100 β-immunoreactivity (Control 2; single S100β staining in inset). Nuclei are stained with DAPI (blue) in all panels. Original magnifications: (B, F) bars indicate 2 and 10 μm, respectively; original magnifications: (E, G) 400x.
Figure 3. Vanishing white matter disease (VWM) astrocytes express oligodendrocytic lineage-specific proteins. (A, B) Double stain for glial fibrillary acidic protein (GFAP) (A; Patient VWM3) or vimentin (B; Patient VWM99) and galactocerebroside (GalC) show dysmorphic astrocytes with homogeneous cytoplasmic GalC immunoreactivity extending far into the processes. (C) In astrocytes from VWM patients, GalC colocalizes with other astrocytic lineage-specific markers, including GFAPδ (Patient VWM44). (D) GFAP-positive cells with astrocyte morphology also express olig2 with a cytoplasmic immunoreactivity pattern (Patient VWM80). (E) Primary culture of sorted glial progenitors
neurologic controls. White matter astrocytes in AXD were diffusely GFAPδ-positive (fig. 4E). Small numbers of faintly GFAPδ-positive cells were detected within an MS plaque and in the X-ALD white matter, which were confined to areas of active demyelination and colocalized with reactive astrocytes (data not shown). No GFAPδ-positive astrocytes were found in the GLD case (data not shown).

To explore the possible role of GFAPδ overexpression in the morphology of VWM astrocytes, brain sections were probed for the heat shock protein αB-crystallin.

Astrocytes in the VWM patient samples showed robust immunoreactivity for αB-crystallin; its distribution overlapped with that of GFAP and vimentin (fig. 4D). Glial fibrillar acidic protein δ and αB-crystallin coexpressing astrocytes were detected also in AXD (fig. 4F). No αB-crystallin-positive astrocytes were found in the other neurologic and nonneurologic controls.

Oligodendrocytes in VWM

*Increased density of oligodendrocyte progenitor cells in VWM*

To investigate whether the maturation status of oligodendroglia is perturbed in VWM, we identified cells in the oligodendrocyte lineage by their nuclear expression of olig2 and assessed the proportion of early oligodendrocyte-competent progenitor cells (OPCs) by their coexpression of the α-subunit of the receptor for platelet-derived growth factor (PDGFRα). The labeling index of PDGFRα varied inversely with age in controls, being highest in the white matter from the 4.5-month-old infant. However, in VWM white matter, the numbers of PDGFRα-positive cells were constant, irrespective of patient age, and were significantly increased (2.2-fold) versus controls (fig. 5A). Positivity for PDGFRα identified cells with small cell bodies that give rise to multiple branched fine radial processes (fig. 5C,E-G), consistent with the morphology of OPCs in the white matter. Double staining for PDGFRα and Ki-67 showed that these immature oligodendrocyte lineage cells were continuing to proliferate in the VWM patient white matter at a higher rate than

*Figure 3 (cont.).* shows a cell with both nuclear and cytoplasmically translocated olig2 and morphology and CD44-immunoreactivity, consistent with an astrocyte progenitor; 2 oligodendrocyte lineage-committed cells only express nuclear olig2 (Patient VWM576). In all panels, the nuclei are stained with DAPI (blue). Original magnifications: (A, E) 200x; (B-D) 400x.
Figure 4. Vanishing white matter disease (VWM) astrocytes overexpress glial fibrillary acidic protein δ (GFAPδ) and αB-crystallin. (A) Dysmorphic astrocytes in the white matter of a VWM patient (VWM3) are strongly GFAPδ-positive. Immunoreactivity extends far into the processes (inset). (B) Higher amounts of GFAPC are detected by Western blot of white matter lysates from 3 VWM patients versus age-matched non-neurologic disease controls. The upper blot shows GFAPC (49 kd) and the lower blot, the loading control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (37 kd). (C) Quantitative PCR of the same patients and controls confirms that GFAP expression is significantly increased at the mRNA level (p=0.005, error bars=SDs). The abundance of the mRNA transcripts of the predominant isoform GFAPα is comparable (p=0.4). (D) Dysmorphic VWM astrocytes also overexpress αB-crystallin, shown colocalizing with the astrocytic marker vimentin (Patient VWM3). (E, F) Similar GFAPδ (E) and αB-crystallin (F) expression is found in the white matter of the Alexander disease (AXD)
in controls (fig. 5B).

Because PDGFRα alone did not stain a proportion of cells in the VWM white matter, tissue sections were additionally probed against another nominal OPC marker, chondroitin sulfate proteoglycan (NG2). There was also a significantly increased density (4.2-fold) of NG2-positive OPCs in the VWM versus control white matter (fig. 5A). The anti-NG2 antibody also labeled other cell types, that is, pericytes lining blood vessels; therefore, NG2-positive OPCs were identified by both NG2 positivity and morphology. As for PDGFRα, NG2-positive OPCs had small round cell bodies with thin processes (fig. 5D), whereas pericytes along the blood vessels are elongated cells without processes.

**Discussion**

*Astrocytic immaturity: a clue for meager gliosis and failing scar formation in VWM*

Reactive gliosis is limited in the white matter of VWM patients, which seems to be disproportionate to the often dramatic extent of local tissue loss. This relative lack of proportionate gliosis may contribute to the development of the cavitating lesions typical of the disease.

To investigate the mechanistic basis for this observation, we first evaluated the proliferation index of VWM astrocytes. Several lines of evidence suggest that mature astrocytes can reenter the cell cycle. Thus, parenchymal astrocytes, like persistent glial progenitor cells, may be a source of new glia after CNS injury (Carmen et al., 2007). We found that a significant proportion of astrocytes are proliferating in VWM, suggesting that the local generation of new astrocytes is not impaired in vivo. The same result was observed in vitro after mechanical stress. A previous study using cultured cells derived from VWM brain had reported that the generation of new astrocytes in vitro might be severely compromised (Dietrich et al., 2005). The discordance between these previous observations in culture and our in vitro results may derive from unique aspects of the response of VWM cells to the culture environment in basal conditions and after stress.

*Figure 4 (cont.).* Patient, with strong perivascular αB-crystallin immunoreactivity consistent with Rosenthal fibers (F). Note the normal morphology of AXD astrocytes (E). In all pictures, nuclei are stained with DAPI (blue). Original magnifications: (A, E, F) 200x; (A, inset) 630x; (D) 400x.
To evaluate whether the deficient reactive gliosis in VWM might be related to a failure in the maturation of newly generated astrocytes (Buffo et al., 2008), we assessed the expression patterns of their intermediate filaments. Astrocytes express a predictable, sequential pattern of intermediate filaments during differentiation, which is recapitulated in response to injury. The human GFAP promoter drives GFAP expression at very early stages of astrocyte development. Immature astrocytes also express nestin and vimentin, whereas only vimentin is coexpressed with GFAP in mature cells (Middeldorp et al., 2010; Eliasson et al., 1999). As such, the concurrent induction of nestin and upregulation of GFAP and vimentin are hallmarks of reactive astrogliosis, both experimentally and in disease (Pekny & Pekna, 2004). In this, the induction of nestin expression has been regarded as indicative of astrocytic dedifferentiation (Shibuya et al., 2002). We also assessed the expression of the Ca2+- and Zn2+-binding protein S100β. Expression of this protein in the adult brain defines a developmental stage, in which astrocytes lose their stem cell potential and reduce migratory behavior to acquire a terminally differentiated phenotype (Raponi et al., 2007; Donato et al., 2009). We found that VWM astrocytes, like reactive astrocytes, robustly express nestin, vimentin and GFAP but express little immunodetectable S100β, all suggesting an immature phenotype. The relative ratios and net abundance of total GFAP and GFAPα mRNA transcripts were unchanged. Together, these data suggest that VWM astrocytes are blocked from developmental maturation, thereby impairing reactive gliosis. The failure of VWM astrocytes to upregulate the predominant GFAP isoform GFAPα may have significant functional consequences for their cytoskeletal architecture, ability to scar cavitated tissue, and interlaminar signaling.

**VWM astrocytes express oligodendrocyte-specific markers**

In their in vitro study of astrocytic dysfunction in cultures derived from a VWM patient, Dietrich et al. (2005) reported that, after exposure to bone morphogenic protein 4, which typically induces astrocytic phenotype from phenotypically unrestricted progenitor cells, 30% to 50% of the GFAP-positive cells showed an aberrant mixed lineage phenotype by also expressing the oligodendrocyte-specific galactolipid GalC. The same observation was made after RNA-interference targeting of EIF2B5 in normal human glial progenitor cells (Dietrich et al., 2005). These data were interpreted as further confirmation of major astrocyte
Figure 5. Increased density of oligodendrocyte progenitor cells (OPCs) in the white matter of Vanishing white matter (VWM) patients. (A) There are higher numbers of OPCs in the white matter of VWM patients versus controls as revealed by both platelet derived growth factor receptor α (PDGFRα) (p<0.0001) and NG2 immunoreactivity (p=0.001) (n=5 patients, 3 controls; bars=SD). (B) Cell counts of Ki-67-positive OPCs show increased proliferation in VWM patients versus nonneurologic controls (p=0.01) (n=5 patients, 3 controls; bars=SD). (C-G) Both PDGFRα and NG2 antibodies identify cells with nuclear olig2-positivity (C, Patient VWM295; D, Patient VWM99) and little cytoplasm with multiple short branches (E-G, Patient VWM367; PDGFRα). The olig2 antibody also labels cells with cytoplasmic immunoreactivity (uppermost cell in C; lower cell in D). These cells are presumably immature astrocytes. Nuclei in (C) and (D) are stained with DAPI (blue). Original magnifications: (C) see bar; (E) 200x; (D, F, G) 400x.
abnormalities in VWM. We demonstrate here that VWM astrocytes are also strongly GalC-positive in situ, showing a diffuse immunoreactivity in the cytoplasm of the cell body and processes. No vesicular distribution of GalC was observed in VWM, as would be expected for astrocytes that have ingested myelin fragments from surrounding degenerating oligodendrocytes. This was probably the case in the few GalC-positive astrocytes we detected in the demyelinating lesions of the MS and X-ALD patients. Conversely, the diffuse GalC distribution in VWM astrocytes is compatible with endogenous synthesis of GalC. Supporting this possibility is the previous finding of an increased specific activity of UDP-galactose:ceramide galactosyltransferase, the enzyme responsible for the final step in GalC synthesis, in the cerebellar white matter of a VWM patient despite a severe paucity of myelin (Rodriguez et al., 1999). Similarly, the coexpression of GFAP and GalC by many astrocytes in GLD (which is caused by deficient activity of the GalC catabolic enzyme galactocerebroside β-galactosidase) may also be consistent with our interpretation (A. Suzuki, unpublished observation).

GalC-expressing astrocytes are not unique to VWM. Glial cells expressing GFAP and a variety of oligodendrocytic markers, including GalC, have been described in normal and disease environments both in vitro and in vivo. Interestingly, many of the pathologic conditions in which this phenomenon has been noted are disorders of myelin. For example, GFAP/GalC coexpressing cells were detected in glial cultures from shiverer mice, which have a partial deletion of the myelin basic protein gene (MBP) (Dyer et al., 2000), as well as in the cat optic nerve after experimental demyelination (Carroll et al., 1987). GalC-positive astrocytes were also reported in tissue sections from patients with the pigmentary type of orthochromatic leukodystrophy (Möller et al., 2003). Glial fibrillary acidic protein-positive cells expressing other oligodendrocyte or myelin markers, including sulfatide/O4, myelin basic protein, and myelin/oligodendrocyte specific protein, have been detected in sections of normal adult rat brain (Pernber et al., 2002), hypomyelinated brains of shiverer, quaking, and phenylketonuria mice (Dyer et al., 2000), and in the spinal cord of mice with viral-induced demyelination (Godfraind et al., 1989). These cells have been variably interpreted as either GFAP-expressing oligodendrocytes (Dyer et al., 2000) or as myelin gene-expressing astrocytes (Carroll et al., 1987; Pernber et al., 2002; Godfraind et al., 1989). On the basis of their promiscuous antigenicity and broad distribution, cells with this phenotype have been postulated to represent glial or astrocyte progenitors (Pernber et al., 2002), or to identify a subpopulation of aberrant mature glia that have initiated the concurrent synthesis of both astrocytic and oligodendrocytic determinants (Carroll et al., 1987; Godfraind et al., 1989).
The finding of defective astrocytic maturation in the white matter of VWM patients is further supported by the presence in vivo and in vitro of cells coexpressing nominally mature astrocytic markers with the transcription factor olig2, and with the latter’s translocation to the cytoplasm. Olig2 in glial cells inhibits astrocyte differentiation, thereby allowing the cells to maintain an undifferentiated state or to develop into myelinating oligodendrocytes (Zhou & Anderson, 2002). The loss of olig2 in rodent glial precursor cells is typically a necessary step preceding astrocytic differentiation, both developmentally (Marshall et al., 2005; Cai et al., 2007) and after injury (Cassiani-Ingoni et al., 2006; Magnus et al., 2007). This activity of olig2 is regulated by its movement between the nucleus and the cytoplasm. Cytoplasmic translocation functionally inactivates olig2 by sequestering it from its nuclear targets. Our finding of cytoplasmic translocation of olig2 suggests that in humans, as in rodents, the removal of olig2 from the nuclear compartment might also be a necessary condition for astrocytic differentiation after damage. Together, these observations also suggest a dual origin of new astrocytes in VWM, which may arise from both resident astrocytes resuming proliferation in response to injury and from adult glial progenitor cells, which commit to astrocytic fate after the cytoplasmic translocation of their nuclear olig2.

**Dysmorphic astrocytes in VWM display abnormal intermediate filaments properties**

The abnormal morphology of VWM astrocytes has been invoked to explain the extensive white matter cavitation and limited gliosis of VWM tissue (Dietrich et al., 2005). In the affected white matter, astrocytes have coarse blunt processes instead of the fine arborizations seen in controls (van der Knaap et al., 2006). We found that VWM white matter astrocytes overexpress both GFAPδ and αB-crystallin, features that may explain their abnormal morphology and dysfunctional status.

Glial fibrillary acidic protein δ results from alternative splicing of the GFAP gene, which replaces the 2 final exons of the predominant isoform GFAPα with an alternative terminal exon (Condorelli et al., 1999; Nielsen et al., 2002). It is specifically expressed by a subpopulation of astrocytes located in the subpial zone along the brain surface and by the astrocytes lining the ependymal layer around the ventricles, including the neurogenic astrocytes in the subventricular zone of the developing and adult brain (Middeldorp et al., 2010; Roelofs et al., 2005; van der Berge et al., 2010). In normal adult gray and white matter parenchyma, GFAPδ represents only a small fraction of total GFAP (Roelofs et al., 2005; Perng et al., 2008). However, in reactive astrocytes, both GFAPα and GFAPδ are upregulated,
although the ratio of the 2 transcripts remains unchanged (Roelofs et al., 2005). It is only under these conditions that GFAPδ becomes detectable with immunohistochemical methods. This is consistent with the finding of only few weakly GFAPδ-positive cells in diseases with reactive gliosis, including Alzheimer disease (Roelofs et al., 2005), MS plaques (Roelofs et al., 2005) (current study), and actively demyelinating areas in X-ALD (current study). In contrast, VWM white matter astrocytes manifest significant overexpression of both GFAPδ protein and mRNA, whereas the corresponding levels of GFAPα are not increased. Diffuse astrocytic GFAPδ immunolabeling is also found in AXD (Perng et al., 2008) (current study), a leukoencephalopathy caused by GFAP gene mutations (Brenner et al., 2001), in which the inability to form proper astrocytic GFAP networks coexists with incomplete maturation of astrocytes and insufficient myelin formation (Mignot et al., 2004). In vitro assays show that the overexpression of GFAPδ and the expression of the AXD-causing GFAP mutation R416W both result in the accumulation of assembly incompetent intermediate filament proteins that collapse the endogenous GFAP network and increase the levels of the protein chaperone αB-crystallin (Roelofs et al., 2005; Perng et al., 2008). Vanishing white matter astrocytes, like AXD astrocytes (Mignot et al., 2004) (current study), are strongly αB-crystallin-immunopositive, suggesting that the disturbed GFAPδ/GFAPα ratio may perturb filament-filament interactions in VWM astrocytes as well, thereby contributing to their aberrant morphology. Moreover, as postulated for AXD (Mignot et al., 2004), the resulting cytoskeletal defects could lead to astrocytic functional impairment, thus leading to widespread effects on other CNS cell types. Because the presence of normal astrocytes is required for endogenous glia progenitor cells to mature into oligodendrocytes and initiate myelination (Talbott et al., 2005), astrocytic dysfunction in VWM might impede myelin formation, maintenance, or both.

*Increased density of OPCs: a hint to hypomyelination in VWM*

Vanishing white matter pathology is characterized by a substantial increase in the numbers of oligodendrocytes, both around cavitating lesions and in less affected white matter (van der Knaap et al., 2006; Rodriguez et al., 1999; van Haren et al., 2004; Francalanci et al., 2001). This oligodendrocytosis is associated with paucity of myelin. To investigate whether this may reflect an oligodendrocytic maturation arrest leading to an increase in immature premyelinating cells, we assessed the maturation status of these cells by means of PDGFRα and NG2 expression. PDGFRα is a receptor for PDGF, a potent mitogen, survival, and differentiation
factor for oligodendrocytes (Grinspan, 2002), currently considered to be the most reliable OPC marker in the adult brain in vivo (Grinspan, 2002; Zhang et al., 2000). NG2 is a chondroitin sulfate proteoglycan on the membrane of OPCs; its expression begins slightly later than that of PDGFRα (Nishiyama et al., 2009). Both markers colocalize in OPCs in vitro (Nishiyama et al., 2009; Rivers et al., 2008; Zhu et al., 2008) and in vivo (He et al., 2009; Wilson et al., 2006). We found that white matter in VWM disease contains significantly increased numbers of OPCs, disproportionate to the numbers of mature myelinating oligodendrocytes.

Oligodendrocytes are the cell population in the CNS with the most significant turnover, and as such, all stages of precursor cells exist through adult life (Nishiyama et al., 2009; Dawson et al., 2003). In the healthy rodent, most adult-born oligodendrocytes differentiate from local OPCs (Dimou et al., 2008), consistent with the observation that adult OPCs divide but their numbers do not increase (Rivers et al., 2008). In response to injury, OPCs form a reactive glial population that undergoes hypertrophy and mitosis (di Bello et al., 1999; Levine et al., 2001). Adult OPCs can efficiently generate new mature oligodendrocytes in early MS lesions (Nishiyama et al., 2009; Raine & Wu, 1993), as well as after acute experimental demyelination (Gensert & Goldman, 1997; Watanabe et al., 2002). However, OPC proliferation also occurs chronically in white matter of mice with genetic myelination defects as shiverer (Bu et al., 2004) and jimpy (Wu et al., 2000), despite elevated cell density. This has been shown to be closely associated with lack of intact myelin (di Bello et al., 1999). A similar mechanism may operate in VWM, in which both lack of myelin and myelin vacuolation with focal areas of uncompacted myelin close to the axonal membrane are observed (Rodriguez et al., 1999).

The increase in OPC numbers detected in VWM white matter by NG2 labeling was higher than that by PDGFRα labeling. Platelet-derived growth factor receptor α-positive cells exhibit numbers consistent with NG2-positive cells at early developmental stages (He et al., 2009) but are considerably less abundant than the NG2-positive population at older ages (He et al., 2009; Wilson et al., 2006). In this respect, our results are in agreement with earlier reports, showing that the distribution of PDGFRα-positive cells within the adult human normal and MS white matter is similar to that of NG2-positive cells, but in numbers that are at least 50% lower (Wilson et al., 2006).

As the ratio of adult OPCs to oligodendrocytes in the normal white matter is approximately 1:4 (Levine et al., 2001) our finding of a 2- to over 4-fold increase is
consistent with estimates of the number of new oligodendrocytes that are required for repair (di Bello et al., 1999). However, despite increased OPC numbers, no remyelination is observed in VWM. We could not directly investigate whether proliferating OPCs can differentiate into myelin-forming cells in VWM autopsy tissue. Downregulation of NG2 and PDGFRα antigens before terminal differentiation of OPCs into mature oligodendrocytes (Zhang et al., 2000; Nishiyama et al., 2009; Zhu et al., 2008) precludes such verification by double immunohistochemical labeling. However, the finding of significantly reduced amounts of myelin structural proteins and lipids previously detected by immunohistochemistry and direct quantification (Shiffmann et al., 1994; Rodriguez et al., 1999; Francalanci et al., 2001; Tedeschi et al., 1995) suggests that only few OPCs can differentiate into myelinating oligodendrocytes in the affected white matter of VWM patients.

Conclusions

Our data suggest that a combination of astrocytic immaturity and cytoskeletal dysfunction with increased numbers of maturation-deficient glial progenitor cells may explain the striking concurrence of compensatory oligodendrocytosis, myelin paucity, and diminished reactive gliosis that characterize the neuropathologic findings in VWM.

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