The Unfolded Protein Response in Vanishing White Matter Disease

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ABSTRACT
Leukencephalopathy with vanishing white matter (VWM) is an autosomal-recessive disorder in which febrile infections may provoke major neurologic deterioration. Characteristic pathologic findings include cystic white matter degeneration, foamy oligodendrocytes, dysmorphic astrocytes and oligodendrocytes, oligodendrocytosis, and apoptotic losses of oligodendrocytes. VWM is caused by mutations in eukaryotic initiation factor 2B (eIF2B). eIF2B plays an important role in the regulation of protein synthesis. Mutant eIF2B may impair the ability of cells to regulate protein synthesis in response to stress and perhaps even under normal conditions. An overload of misfolded proteins in the endoplasmic reticulum activates the unfolded protein response (UPR), a compensatory mechanism that inhibits synthesis of new proteins and induces both prosurvival and proapoptotic signals. We have studied the activation of the UPR in VWM through the immunohistochemical expression of its upstream components PERK and phosphorylated eIF2α (eIF2αP) and combined immunohistochemical and Western blot analysis of the downstream effector proteins activating transcription factor-4 (ATF4) and C/EBP homologous protein (CHOP) in 4 VWM brains and 3 age-matched controls. We demonstrate activation of the UPR in glia of patients with VWM. Our findings may point to a possible explanation for the dysmorphic glia, the increased numbers of oligodendrocytes, and the apoptotic loss of oligodendrocytes in VWM.

KEY WORDS: Apoptosis, Childhood ataxia with central nervous system hypomyelination (CACH), eIF2B, Unfolded protein response (UPR), Vanishing white matter (VWM).

INTRODUCTION
Leukencephalopathy with vanishing white matter (VWM), also termed childhood ataxia with central nervous system hypomyelination (CACH), is an autosomal-recessive white matter disorder (1, 2). Its clinical course is chronic and progressive with intermittent episodes of major and rapid neurologic deterioration. These episodes are provoked by minor head trauma and particularly febrile infections. VWM is caused by mutations in any of the 5 genes encoding the subunits of the eukaryotic initiation factor (eIF) 2B (eIF2B) (3, 4).

eIF2B plays an essential role in the initiation of protein synthesis by catalyzing the GDP-GTP exchange on eIF2 to enable binding of methionyl-transfer-RNA (Met-tRNA) to the small ribosomal subunit (5). The ability to regulate the activity of eIF2 represents a crucial protective mechanism of cells in response to a variety of stress conditions, including fever. Hyperthermia leads to denaturation of proteins contributing to cell dysfunction and death. eIF2B is the main factor to downregulate protein synthesis in hyperthermia (6). Downregulation of eIF2B is mainly achieved by phosphorylation of eIF2α, one of the 3 subunits of eIF2. When phosphorylated at its α-subunit, eIF2 binds irreversibly to eIF2B and in this way inactivates it (7). In the brain, the concentration of eIF2 is 4 to 5 times that of eIF2B; consequently, when approximately 20% to 25% of total eIF2α is phosphorylated, the synthesis of new proteins is substantially inhibited (8, 9).

Shutdown of protein synthesis protects cells against the accumulation of misfolded and denatured proteins in the endoplasmic reticulum (ER) (10). Mutant eIF2B in VWM may impair the ability of cells to regulate protein synthesis, especially in response to cell stress and possibly even under normal physiological conditions.

An overload of misfolded or denatured proteins in the ER activates the unfolded protein response (UPR). The UPR is an integrated intracellular signaling pathway that transmits information about the protein folding status in the ER lumen to the cytoplasm and the nucleus. The UPR leads to 1) inhibition of protein synthesis at a translational level by inactivation of eIF2B to immediately relieve the protein load on the organelle, 2) transcriptional induction of genes that code for ER-resident chaperones and enzymes to abate the effects of ER stress, and 3) induction of genes involved in ER-associated degradation. An activated UPR can induce both prosurvival and proapoptotic signals. If the protein-folding defect is not corrected, cells undergo apoptosis (11, 12).
Inhibition of protein synthesis is achieved through activation of the ER-resident kinase PERK. PERK couples stress signals initiated by protein misfolding in the lumen of the ER to phosphorylation of Ser51 on the α-subunit of eIF2α (13).

Apoptotic pathways, involving activation of activating transcription factor-4 (ATF4) and its downstream target C/EBP homologous protein (CHOP), can also be directly activated by decreased activity of eIF2B. Small upstream open reading frames in the 5 untranslated region in the mRNA of ATF4 will lead to increased expression of this transcription factor under conditions in which eIF2B activity is reduced (14).

Activation of the UPR has been implicated in the pathophysiology of several neurologic disorders (15, 16). In Pelizaeus-Merzbacher disease, the disease severity is modulated by the UPR (16). A recent study in our laboratories has demonstrated apoptosis and abnormal proliferative and ant apoptotic phenomena in oligodendrocytes of VWM (17). In the present study, we wanted to test whether the UPR is activated in the ER of patients with VWM by immunohistochemical staining and Western blot analysis of various components of the UPR, including activated PERK and phosphorylated eIF2α (eIF2αP) as well as the downstream effector proteins ATF4 and CHOP.

MATERIALS AND METHODS

Patients

Brain tissue from 4 patients with VWM and 3 controls was included in our studies. At the time of autopsy, all patients’ next of kin consented to the use of patient tissues for research. The 4 patients identified as having VWM were confirmed by genetic testing. Controls were selected with an attempt to age-match patients and without significant or confounding neuropathologic findings at the time of autopsy. The ages of the VWM patients were 3 months, 6 and 12 years, whereas the ages of controls were 9 months, 2 and 9 years. Table 1 outlines the clinical and demographic information of patients and controls. The brains were fixed for a minimum of 7 days in formalin (phosphate-buffered, 4% formaldehyde) before they were sectioned, and selected portions of each brain, including both cerebral white matter and cortex, were embedded in paraffin. Tissue sections were cut from the brain, including both cerebral white matter and cortex, were embedded in paraffin. Tissue sections were cut from the paraffin blocks and stained with routine neuropathologic techniques. Additionally, brain tissue from patient 1 was frozen immediately in liquid nitrogen at the time of autopsy, and subsequently lyzed and used for Western blotting as described subsequently.

Immunohistochemical Staining

Cerebral white and gray matter of all patients and controls was stained with primary antibodies to the following epitopes: carbonic anhydrase II (CA II; Rockland, Gilbertsville, PA) diluted 1:15000; glial fibrillary acidic protein (GFAP; DAKO, Carpinteria, CA) diluted 1:1000 with antigen retrieval; KP1 (CD68; DAKO) diluted 1:400; eIF2αP (pSer51) (Sigma-Aldrich, St. Louis, MO) diluted 1:200. Negative (by omitting the primary antibody) and positive controls were performed with each run. The following positive immunostaining controls were used for the antibodies not in standard use: pancreatic islet delta cells for phospho-PERK (18), degenerating neurons in Alzheimer disease hippocampus for eIF2αP (19), hormone-producing cells in anterior pituitary for ATF4 (20), and oligodendrocytes in Pelizaeus-Merzbacher disease brain for CHOP (16).

An indirect immunohistochemical technique using the streptavidin–biotin system was carried out using 3-aminomethylcarbazole (AEC; ScyTek Laboratories, Logan, UT) as chromogen. Double immunostaining for CHOP or ATF4 and CAII or GFAP was restricted to brain samples of patients 1 and 3. It included the addition of another Chromagen, Vector SG substrate for peroxidase (Vector Laboratories, Burlingame, CA), to the GFAP antibody. A MACH 3 system was used (Biocare, Walnut Creek, CA) when double immunostaining included the CAII antibody.

Quantification

The total number of cells, both labeled and unlabeled, were counted (JPvdV) in at least 10 standardized microscopic fields of 0.1 mm² each (defined by an ocular grid), randomly chosen in a relatively small section of preserved cerebral white matter. For each tissue section stained for phospho-PERK, eIF2αP, ATF4, and CHOP, labeled cells were counted individually and expressed as a percentage of the total number of cells. The results from these counts were averaged for each patient. The intensity of the cytoplasmic and nuclear staining was evaluated on a 3-point scale: 0 = none, + = mild, and ++ = moderate to intense.

SDS-PAGE and Western Blotting

White and gray matter from control 1 and patient 1 were separated at –20°C. Approximately 1 mg of material was taken up in 1.5 mL NP40-lysis buffer (20 mM Hepes, 50 mM KCl, 25 mM β-glycerophosphate, 0.2 mM EDTA, 1% NP40) containing a protease inhibitor cocktail (protease inhibitor cocktail tablets; Roche, Indianapolis, IN) and 1 mM DTT. The material was homogenized using a pellet pestle mortar with

<table>
<thead>
<tr>
<th>TABLE 1. Demographic Data</th>
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<tr>
<td>Subject</td>
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<tr>
<td>Patient 1</td>
</tr>
<tr>
<td>Patient 2</td>
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<tr>
<td>Patient 3</td>
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<tr>
<td>Patient 4</td>
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<tr>
<td>Control 1</td>
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<tr>
<td>Control 2</td>
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<tr>
<td>Control 3</td>
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PMI, postmortem interval.
polypropylene pestles (Sigma-Aldrich). Extracts were centrifuged for 20 minutes at 14,000 g (4°C). Supernatants were aliquoted and stored at −80°C. SDS-PAGE sample buffer was added to the samples, which were then heated for 20 minutes at 95°C, run on 12.5% SDS-polyacrylamide gels, and transferred onto PVDF membrane (Immobilon P; Millipore, Billerica, MA). The blots were incubated with antibodies against ATF4 and CHOP (both from Santa Cruz Biotechnologies) and α-tubulin (Sigma-Aldrich). Proteins were visualized using ECL Western Blot detection reagent (Amersham, Piscataway, NJ). As positive controls for the ATF4 and CHOP antibodies lymphoblasts (EBV-immortalized lymphocytes derived from healthy volunteers), either untreated (−) or incubated with 10 μg/mL Tunicamycin (Tm) (Sigma-Aldrich) for 4 hours (+), were used.

**RESULTS**

Immunostaining for all antibodies was found in the white matter of the 4 patients with VWM. The intensity of immunoreactivity and number of labeled cells varied considerably between the patients, with the most intense and extensive labeling in patient 1. Importantly, labeling was absent in the cortex of all patients with VWM and in both the white matter and cortex of 2 controls. The only exception was that mild immunoreactivity for ATF4 and CHOP was observed in scattered white matter cells and in some cortical neurons in control 2. The percentage of immunostained white matter cells in patients and controls is indicated in Table 2. The VWM white matter results are discussed in more detail subsequently.

Like in the pancreatic islets cells (Fig. 1A), within the white matter of patient 1, intense cytoplasmic staining for phospho-PERK was found in numerous cells with the morphologic features of oligodendrocytes (dense round nucleus, often with a small nucleolus, and perinuclear halo) as well as in dysmorphic astrocytes (with large cytoplasm and blunt broad processes rather than their typical delicate arborizations) (Fig. 1B). Oligodendrocytes with abundant or foamy cytoplasm, characteristic of VWM (24, 25), also were immunoreactive. Phospho-PERK expression was detected in the same, but fewer, cells in patients 2, 3, and 4.

Cytoplasmic eIF2αP expression was detected in few glial cells of patient 2 (Fig. 1C). Staining was also evident in some endothelial cells. Labeling was rare in patients 1, 3, and 4.

Comparable to ATF4-staining in anterior pituitary cells (Fig. 1D), many cells scattered throughout the cerebral white matter stained positively for ATF4 in patients 1 and 4 (Fig. 1E, F). These cells were morphologically consistent with both oligodendrocytes and astrocytes. Most of the positive cells had abundant cytoplasm. These cells with large cytoplasm stained positively for either CAII (not shown) or GFAP (Fig. 1G) and were morphologically distinct from CD68-positive macrophages (Fig. 1H). A lower number of immunoreactive cells were seen in patients 2 and 3. ATF4 staining was primarily cytoplasmic.

Immunohistochemical staining of CHOP was found to be comparable to the ATF4 and phospho-PERK staining in patients 1, 2 and 3. Mild immunoreactivity was observed in a lower number of cells in patient 4. Immunoreactivity with anti-CHOP was primarily located in nuclei (Fig. 1I).

Double immunostaining in patients 1 and 3 for CHOP or ATF4 and CAII or GFAP confirmed that both oligodendrocytes and astrocytes expressed ATF4 and CHOP (not shown).

Western blotting of cell extracts of white and gray matter from control 1 and VWM patient 1 confirmed the immunohistochemical data. ATF4 and CHOP expression was clearly detectable in the white matter of the VWM patient (Fig. 2). In the white matter of the control, a very faint band was seen at the height of ATF4.

**DISCUSSION**

For unknown reasons, the central white matter appears to be selectively affected in VWM, although it has been shown recently that cataracts and involvement of ovaries, liver, kidneys, and pancreas may accompany the leukoencephalopathy (21, 22). There is increasing evidence that glial cell dysfunction is central to the pathophysiology of VWM. At autopsy, most patients with VWM reveal diffusely rarefied to cavitated deep cerebral white matter with profound losses of oligodendrocytes, myelin sheaths, and axons, accompanied by a feeble astrogliosis and macrophage response. A paradoxically increased cellular density of oligodendroglia is found in the relatively preserved white matter areas such as the subcortical white matter and the anterior commissure. Many oligodendrocytes have unusually abundant cytoplasm; astrocytes are also dysmorphic (2, 23–26). Recently, in a study that focused on the oligodendrocytic pathology in VWM, we demonstrated that oligodendrocytes are being subjected to conflicting proliferative, cell death, and cell survival signals and that they are dying by apoptosis, perhaps in conjunction

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**TABLE 2. Percent White Matter Cells Labeling with Respective Stains**

<table>
<thead>
<tr>
<th></th>
<th>Phosho-PERK Percent, Intensity</th>
<th>eIF2αP Percent, Intensity</th>
<th>ATF4 Percent, Intensity</th>
<th>CHOP Percent, Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td>9, ++</td>
<td>1, +</td>
<td>9, ++</td>
<td>3, ++</td>
</tr>
<tr>
<td>Patient 2</td>
<td>2, +</td>
<td>5, +</td>
<td>2, +</td>
<td>4, +</td>
</tr>
<tr>
<td>Patient 3</td>
<td>1, +</td>
<td>0.5, +</td>
<td>1, ++</td>
<td>1, ++</td>
</tr>
<tr>
<td>Patient 4</td>
<td>0.5, +</td>
<td>1, +</td>
<td>16, ++</td>
<td>0.5, +</td>
</tr>
<tr>
<td>Control 1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Control 2</td>
<td>0</td>
<td>0</td>
<td>1, +</td>
<td>2, +</td>
</tr>
<tr>
<td>Control 3</td>
<td>0</td>
<td>0</td>
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<td>0</td>
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</table>

The intensity reflects the amount of staining on a 3-point scale: 0 = none, + = mild, and ++ = moderate to intense.
with major neurologic crises (17). Apoptosis of oligodendrocytes in the VWM brain has also been observed by others (26).

Our present immunohistochemical data demonstrate activation of several of the components of the UPR in glial cells of VWM cerebral white matter, both oligodendrocytes and astrocytes. In these cells, PERK is activated, whereas phosphorylation of eIF2α and expression of ATF4 and CHOP are specifically increased. Western blotting confirmed the increased expression of ATF4 and CHOP in VWM white matter. The UPR in macrophages may also be activated. If so, this is far less common.

Activation of the UPR components varied in our small sample of patients with VWM with the most apparent activation in patient 1. This patient experienced prolonged terminal brain hypoxia, which causes changes in gene expression. Brain samples from individuals with prolonged agonal stress (and with low pH in the brain) exhibited higher...
expression of genes involved in the stress response, apoptosis, and inflammation control than brain samples from individuals who experienced brief deaths (and generally had normal pH) (27). However, it is not likely that terminal hypoxia-induced UPR fully explains the better labeling in patient 1, because only mild activation of the UPR was observed in one control, who also had a prolonged agonal state with respiratory failure leading to hypoxia in the brain. Furthermore, after global brain ischemia, only the most vulnerable CA1 hippocampal neurons, not those in cerebral cortex, demonstrated activation of UPR (28).

Labeling for UPR components was absent in the 2 other controls, and Western blotting of cell extracts of white matter of control 1 showed only a very faint band at the height of ATF4. ATF4 exists in multiple forms, leading to an apparent smear on Western blotting, and this smear is absent in the white matter of the control. Furthermore, although we cannot completely exclude that this band is indeed ATF4, this band appeared to run slightly different than the lowest form of ATF4.

Sustained low levels of eIF2 activity, related to mutations in eIF2B, may also lead to increased ATF4 levels independent of activation of the UPR. ATF4 mRNA translation is enhanced through a mechanism involving small upstream open reading frames in the 5 untranslated region of ATF4. ATF4 mRNA translation is enhanced through a mechanism involving small upstream open reading frames in its 5 untranslated region (14). Normally, CHOP is expressed at low or undetectable levels (16). Overexpression of CHOP promotes cell cycle arrest or cell death.

However, there is growing evidence that VWM mutations cause decreased activity of eIF2B, resulting in decreased protein production, and that they do not confer temperature sensitivity on cells (30–32). It is, therefore, not clear if and how VWM mutations can lead to accumulation of misfolded proteins in the ER and activation of the UPR. One possible mechanism would be that decreased activity of eIF2B in VWM leads to increased expression of ATF4 related to the upstream open reading frames in the 5 untranslated region of ATF4 mRNA, and subsequently increased expression of CHOP even without activation of the UPR. Evidence has been found that CHOP sensitizes cells to ER stress (33). Febrile infections and minor head trauma may contribute to ER stress in the ER stress-sensitive patients with VWM leading to activation of the UPR and to an imbalance between cell survival and cell death signaling pathways. The UPR induces prosurvival and proapoptotic signals and both are seen in oligodendrocytes and astrocytes in VWM (17). Most likely, the balance between these 2 contradicting signals determines the fate of oligodendrocytes. Why glial cells are the preferentially affected cell type in VWM remains an open question.

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REFERENCES

5. Proud CG. Regulation of eukaryotic initiation factor eIF2B. Prog Mol Subcell Biol 2001;26:95–114