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Gene expression analysis suggests that 1,25-dihydroxyvitamin D_3 reverses experimental autoimmune encephalomyelitis by stimulating inflammatory cell apoptosis

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vitamin D; multiple sclerosis; autoimmune disease; DNA microarray

MULTIPLE SCLEROSIS (MS) is a debilitating autoimmune disease of the central nervous system (CNS). In MS, an abnormal immune response to myelin antigens causes mononuclear cell infiltration, demyelination, oligodendrocyte loss, and axonal degeneration (4). The etiology of MS is uncertain, but the available immunologic, genetic, and epidemiological data suggest that MS develops in genetically susceptible individuals who are exposed to as-yet undefined causal environmental risk factors (53). Genetic heterogeneity exists with respect to MS susceptibility, likely reflecting the action of several genes, each with moderate effects. The MS susceptibility alleles are incompletely penetrant, indicating that environmental risk factors determine whether the disease develops. Identifying these environmental risk factors and how they influence disease development are important goals of MS research.

Several studies have shown that a lack of ultraviolet B (UVB) sunlight exposure is an environmental risk factor for MS (1, 24, 27, 75, 76). Because MS prevalence decreases with increasing UVB exposure, and UVB light is essential for vitamin D_3 synthesis, we have hypothesized that the vitamin D_3 hormone, 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$], may be a natural inhibitor of MS (29). Consistent with this hypothesis, we and others found that 1,25-$(OH)_2D_3$ profoundly inhibited the induction of experimental autoimmune encephalomyelitis (EAE) (11, 13, 46, 49, 51) and rapidly reversed established EAE disease (50). EAE, an induced disease, is a widely used MS model (67). Like MS, EAE occurs in relapsing-remitting or chronic progressive forms, shows strong genetic susceptibility associated with the major histocompatibility complex, reflects environmental triggers related to prior infection, and exhibits white matter lesions associated with T helper type 1 (Th1) cells and gray matter pathology with axonal degeneration.

Although UVB light catalyzes vitamin D_3 synthesis, two further metabolic steps are required to convert this biologically inactive compound into a hormone (54). A constitutive liver enzyme, vitamin D_3-25-hydroxylase (25-OHase), catalyzes a C-25 hydroxylation. Thereafter, an inducible kidney enzyme, 25-hydroxyvitamin D_3-1α-hydroxylase (1α-OHase), catalyzes a C-1 hydroxylation. It is highly significant that microglia (25, 81), neurons (25, 81), and interferon-γ (IFN-γ)-activated macrophages (2, 21, 31, 56) express the 1α-OHase, so the CNS could be a site of active 1,25-$(OH)_2D_3$ synthesis. Furthermore, the myeloid and lymphoid lineage cells, astrocytes, glial cells, neurons, and oligodendrocytes all express the nuclear vitamin D receptor (VDR) (30) that binds 1,25-$(OH)_2D_3$ and forms an activated transcription regulatory complex (28, 39). The presence of the 1α-OHase and the VDR in the CNS led us to hypothesize that given sufficient sunlight exposure and vitamin D_3, the neurons, microglia, and IFN-γ-activated macrophages could produce high levels of 1,25-$(OH)_2D_3$ in the CNS, thereby altering gene expression patterns in a manner that would attenuate inflammation (30).

The purpose of the experiments reported here was to identify the rapid 1,25-$(OH)_2D_3$-mediated gene expression changes in

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the CNS that preceded the resolution of EAE lesions and the reversal of clinical EAE disease (50). We expected that these gene expression changes might provide new insight into how sunlight and vitamin D_3 could inhibit MS. We previously developed an experimental system to study the molecular basis for rapid 1,25-$(OH)_2D_3$-mediated reversal of established EAE (50). EAE was induced, and animals with severe EAE were treated with 1,25-$(OH)_2D_3$ or placebo to begin disease reversal in a synchronous manner. Kinetic studies using this EAE treatment model showed that within 24 h of treatment, the hormone dramatically reduced the inflammatory cells in the CNS lesions (50). These data suggested that the 1,25-$(OH)_2D_3$ may have sensitized the inflammatory cells to apoptosis. Here, we have used the EAE treatment model to analyze how 1,25-$(OH)_2D_3$-mediated activation or repression of vitamin D-responsive genes might reverse EAE. A DNA microarray study examined gene expression patterns in the CNS of hormone-treated and placebo-treated mice with EAE, and a terminal deoxynucleotidyl nicked end labeling (TUNEL) study identified cells undergoing apoptosis. The results strongly suggest that the 1,25-$(OH)_2D_3$ altered gene expression patterns in a manner that would sensitize the inflammatory cells to apoptosis, thereby contributing to lesion resolution in this autoimmune disease model.

MATERIALS AND METHODS

Mice. The B10.PL(73NS)/Sn breeding pairs were originally obtained from the Jackson Laboratory (Bar Harbor, ME). The mice were bred in the Biochemistry Department's pathogen-free mouse colony. The mice were fed laboratory diet 5008 (PMI Nutrition International, Brentwood, MO), containing 3.3 IU vitamin D_3 per gram diet and 1% calcium, and maintained at 23°C with 40–60% humidity and 12:12-h light-dark cycles. The drinking water was provided ad libitum. All animal experimentation was conducted in accord with accepted standards of humane animal care, as outlined in the Ethical Guidelines. The experimental protocols were approved by the Institutional Animal Care and Use Committee.

EAE induction and 1,25-$(OH)_2D_3$ treatment. EAE was induced in adult male mice (age 6–8 wk) with myelin basic protein and pertussis toxin (List Biological Laboratories, Campbell, CA) as described (50), and EAE disease severity was scored daily (11). Mice with an EAE clinical score of 2.5 to 3 were randomized into two groups, and injected intraperitoneally with 200 ng of 1,25-$(OH)_2D_3$ in 0.1 ml of soybean oil (Hunt-Wesson Foods) or soybean oil only as the placebo. The 1,25-$(OH)_2D_3$ stock solution (1 mg/ml absolute ethanol; Tetrionix, Madison, WI) was stored in the dark at −20°C prior to dilution in oil. The placebo- and hormone-treated mice with EAE were euthanized, then perfused with saline, and the spinal cords were removed. Spinal cord samples were obtained from placebo- and hormone-treated mice at 6 h posttreatment. Spinal cords were also obtained from normal male mice. The spinal cords were fixed in 4% paraformaldehyde or flash frozen in liquid nitrogen and stored at −80°C for histopathology or RNA extraction, respectively.

Histopathology. The paraformaldehyde-fixed spinal cords were embedded in paraffin, sectioned longitudinally at 10 μm, stained with Gill's no. 3 hematoxylin and eosin Y (Sigma Diagnostics, St. Louis, MO), and examined using a Zeiss Axioskop microscope equipped with a Plan-Neofluar 20×/0.5 objective. Bright-field images were acquired with AxioVision 3.0 software controlling an Axiocam digital camera. Each of 2 spinal cord sections per mouse was divided into 10 segments, and each segment was further subdivided into 2 parts. Each of the 40 subdivided parts was scored in a blinded fashion as 0 or 1, based on the absence or presence, respectively, of infiltrating inflammatory cells. The histopathology score was recorded as the percentage of the 40 subdivided spinal cord parts that showed a readily identifiable inflammatory cell infiltrate.

Polymerase chain reaction. Total RNA was extracted from frozen spinal cord samples using TRI Reagent (Molecular Research Center, Cincinnati, OH). To assess whether the RNA was intact, a sample was reverse transcribed from an oligo(dT) primer using the Reverse Transcription System (Promega, Madison, WI), and the GAPDH transcripts were PCR amplified using published primers (49). Only RNA samples that yielded a GAPDH amplicon were employed for further studies.

Real-time PCR was performed as described with minor modifications (10). The PCR reactions (25 μl) contained 0.5–1 μg cDNA, 50 nM of each primer, 12.5 μl of 2× SYBR Green PCR Master Mix (PE Applied Biosystems, Foster City, CA), and 2.25 μl H_2O. The amplification was accomplished with a GeneAmp 5700 Sequence Detection Systems instrument (PE Applied Biosystems) programmed for incubations of 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of denaturation for 15 s at 95°C and annealing/extension for 1 min at 60°C.

The GAPDH primers have been published previously (57). The glial fibrillary acidic protein (GFAP) PCR primers were designed using Primer Express software (PE Applied Biosystems) and purchased from Integrated DNA Technologies (Coralville, IA). The GFAP primers were forward 5′-GAA GGT CCG CTT CCT GGA A-3′ and reverse 5′-GGC TCG AAG CTG GTT CAG TT-3′. The CD45 primers were forward 5′-GAA CCC CGG AGC CTA CCA-3′ and reverse 5′-CAG AAC CAT TGG CAG CAT GT-3′. The calpain-2 primers were forward 5′-TAA CCA ACT GAG CCA GGT GTG-3′ and reverse 5′-GCC AGC TTG GTT GTT TCA AG-3′. The caspase-8-associated protein 2 primers were forward 5′-TCA ATG GAG AAA ATC CCA CA-3′ and reverse 5′-AGC TTT TGG TCT CAG TGG CT-3′. To generate a standard curve, cDNA representing each specific amplicon was gel purified and quantified by absorbance at 260 nm. Each real-time PCR included reactions with serially diluted standard cDNA. The standard cDNA copy number, calculated from the absorbance and the dilution was plotted vs. the threshold cycle, C_T. The transcript copy number in each unknown sample was determined from C_T by reference to the appropriate standard curve. The data were calculated as transcript abundance relative to GAPDH as the internal standard.

DNA microarray analysis. Each intact RNA sample was used to synthesize biotin-labeled cRNA exactly as described (45). Each biotin-labeled cRNA sample was hybridized to one microarray (murine U74Av2 GeneChips; Affymetrix) exactly as described (44). The cRNA from *Escherichia coli*, *B. subtilis*, and the P1 bacteriophage served as hybridization controls. The GeneChips were washed, stained first with streptavidin phycoerythrin, then with biotinylated goat antibodies to streptavidin, and finally with streptavidin-phycoerythrin again to amplify the signal. The stained GeneChips were subsequently washed in an Affymetrix fluidics station (model 800101) and scanned at 6-μm resolution on a Hewlett-Packard GeneArray scanner (model 900154).

The gene expression data were analyzed with DNA-Chip Analyzer (dChip) software (47), leading to estimates of log base 2 expression. The log-transformed gene expression data for the 1,25-$(OH)_2D_3$-treated mice (n = 3) were compared with the data for the placebo-treated mice (n = 3). For genes whose expression increased with 1,25-$(OH)_2D_3$ treatment, the fold change was calculated as ($signal_{hormone}/signal_{placebo}$). For genes whose expression decreased with 1,25-$(OH)_2D_3$ treatment, the fold change was calculated as ($signal_{placebo}/signal_{hormone}$). The comparison between the placebo-treated mice (n = 3) and the normal mice (n = 3) was done in an analogous manner. The data have been submitted to the Gene Expression Omnibus (GEO; **http://www.ncbi.nlm.nih.gov/geo**) and can be found under GEO accession numbers GSM13048 through GSM13056 and GSE842.

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Gene expression differences were considered to be statistically suggestive when $P \leq 0.05$ for the Student's t-test and $P \leq 0.058$ for the Mann-Whitney rank sum test (5). Overall ANOVAs comparing all three treatments were conducted but are not reported here. No multiple comparison correction was made for the two tests described above. Instead, we limited our analysis to genes with a $\geq|2.0|$-fold change. As a further guard against false discoveries amongst the 12,488 statistical tests that were conducted on the data set, we used q values to estimate the gene-specific false recovery rate based on the P value derived from the Student's t-test (68). The q value estimates the minimum false discovery rate of one particular gene that has been deemed significant based on the Student's t-test P value (68). Hierarchical cluster analysis was performed using robust multi-array analysis (RMA) with the R software package (**http://bioconductor.org/CRAN**) (36). The genes determined to be significant by the criteria described above were separated into clusters using the average distance method.

In situ apoptosis detection. Spinal cord samples for apoptosis studies were obtained from placebo- and hormone-treated mice at 6 h posttreatment. They were fixed for 24 h in 4.0% paraformaldehyde followed by 70% ethanol in water. The fixed tissue was embedded in paraffin, sectioned longitudinally at 6 μm, and deparaffinized by heating 5 min at 58°C. After a 10-min wash in xylene, the sections were rehydrated by washing sequentially in 100%, 95%, and 70% ethanol in water. Apoptotic cells were detected by TUNEL using a kit (NeuroTACS II in situ apoptosis detection kit; R & D systems, Minneapolis, MN) according to the manufacturer's directions. Apoptosis was evaluated in blinded fashion by determining the percentage of lesions with at least 5% apoptotic cells (as evidenced by intense nuclear TUNEL staining). Bright-field images were acquired as above with a Plan-Apochromat 63×/1.4 oil objective.

RESULTS

Clinical and biological analysis of mice with EAE. Knowledge of rapid gene expression changes in the CNS following hormone administration to mice with EAE could provide important insights into how 1,25-$(OH)_2D_3$ produced locally in the CNS might inhibit MS. To acquire such knowledge on the broadest possible scale, we used DNA microarrays to compare patterns of gene expression in spinal cord cells obtained from hormone-treated and placebo-treated mice with EAE. Severe EAE disease was induced, and mice with a mean clinical score of 2.7 ± 0.2 were randomized to receive an injection of 200 ng 1,25-$(OH)_2D_3$ in oil or oil only as the placebo. This dose was selected for this and prior studies because it effectively inhibited EAE in male mice without causing hypercalcemia (12, 49, 50). It is not clear whether this dose achieved a physiological level of 1,25-$(OH)_2D_3$ in the inflamed CNS, because the 1,25-$(OH)_2D_3$ level in the inflamed CNS is unknown. It could be significantly higher than the 1,25-$(OH)_2D_3$ level in plasma, because the IFN-γ-activated macrophages have a very high 1,25-$(OH)_2D_3$ biosynthetic capacity (22).

Measurements of mouse weight, EAE clinical disease score, and CNS histopathology (Table 1 and Fig. 1), and also flow cytometry of spinal cord cell samples (data not shown), showed that the hormone-treated and placebo-treated mice did not differ with respect to EAE disease parameters or cell populations present in the inflamed CNS at 6 h posttreatment. A histopathological examination of the hematoxylin and eosin-stained spinal cord sections showed no observable differences between the placebo- and hormone-treated samples. Both samples had characteristic hyperacute EAE lesions with an inflammatory cell infiltrate composed primarily of neutrophils and also macrophages and T lymphocytes. Changes in gene expression were evident at this time point, indicating that the hormone had reached the CNS within 6 h. Accordingly, spinal cord RNA samples were collected at 6 h posttreatment for gene expression analysis.

Table 1. *The 1,25-$(OH)_2D_3$- and placebo-treated groups did not differ with regard to EAE disease severity or spinal cord inflammatory cell infiltrate*

Treatment Group	Body Weight, g	Clinical Score	Histopathology, % diseased sections	mRNA	
				CD4+	CD11b+
Placebo	26.7 (2.6)	2.7 (0.2)	61 (9)	826 (43)	168 (77)
1,25-$(OH)_2D_3$	26.7 (1.0)	2.8 (0.3)	53 (11)	923 (83)	217 (77)

Values are means with SD in parentheses. Mice with an experimental autoimmune encephalomyelitis (EAE) clinical score of 2.5–3 were weighed, randomized into two groups, and given an intraperitoneal injection of 200 ng 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] in 0.1 ml oil or oil only as the placebo. The spinal cord samples were collected 6 h later. Spinal cord samples (3/group) for histopathology were formalin fixed, paraffin embedded, sectioned longitudinally, stained, and scored in a blinded fashion as described in MATERIALS AND METHODS. The results represent the percentage of 40 spinal cord segments showing an inflammatory cell infiltrate. Spinal cord cell samples (3/group) were collected, and the RNA was isolated and analyzed for specific transcripts using DNA microarrays.

Prior to gene expression analysis, an RT-PCR for GAPDH was performed to ascertain the RNA sample quality. Three individual RNA samples per group were selected on the basis of a robust GAPDH signal and hybridized to individual GeneChips. To determine the completeness of the hybridizations, the signal intensity ratios of the 5′ and 3′ probes specific for GAPDH, β-actin, and 18S RNA were analyzed. These ratios ranged from 0.8–1.2 for all nine samples, indicating complete hybridization. Neither EAE disease nor hormone treatment affected these genes, as judged by comparisons with normal controls. Thereafter, the remaining gene expression data were log-transformed and analyzed.

EAE disease-induced gene expression changes in the CNS. We first compared the gene expression patterns in the CNS of the placebo-treated mice with EAE to the normal control mice. This acute inflammatory disease appeared to alter the expression patterns of 2.6% of the genes analyzed (327 of 12,488), as determined by applying statistical (Student's t-test $P \leq 0.05$ and Mann-Whitney test $P \leq 0.058$) and fold change ($\geq|2.0|$) criteria for the significance of differences. However, the estimated false discovery rate (q value) was >30% for these P values (68). Therefore, a more stringent criterion of $P < 0.005$ (Student's t-test) was applied to hold the false discovery rate below 30%. By this criterion, 0.8% (95 of 12,488) of the genes had altered expression in EAE disease. Table 2 presents the results for a selected subset of these genes; Supplemental Table S1 presents all of them. (Supplementary Material is available at the *Physiological Genomics* web site.)[1]

The EAE disease increased the expression of many genes associated with antigen processing, presentation, and the im-

[1]The Supplementary Material for this article (Supplemental Tables S1–S4 and Supplemental Figs. S1 and S2) is available online at **http://physiolgenomics.physiology.org/cgi/content/full/00003.2004/DC1**.

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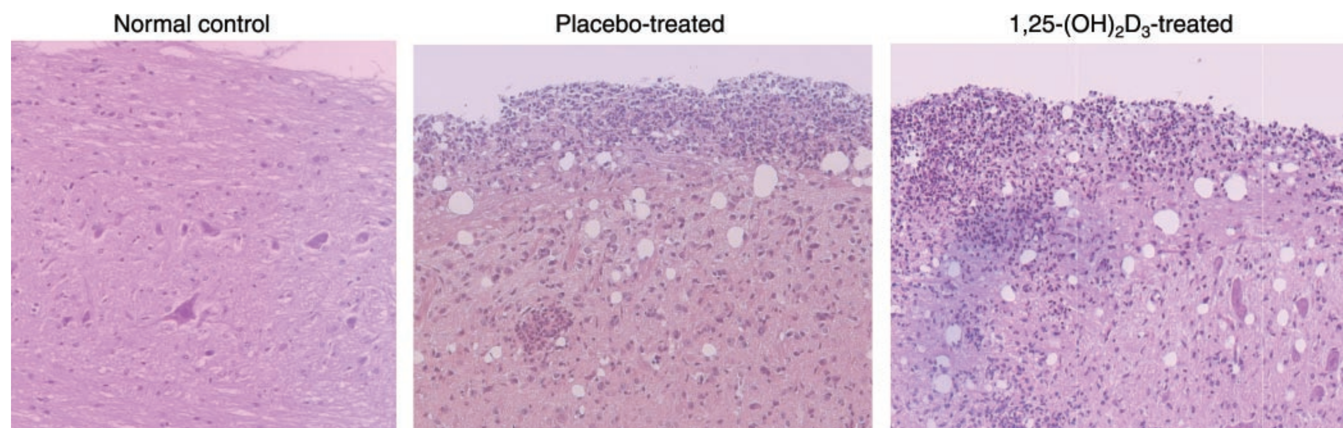

Fig. 1. The spinal cords from placebo-treated and 1,25-dihydroxyvitamin D_3 [$1,25\text{-}(OH)_2D_3$]-treated animals showed equivalent lesion severity and inflammatory cell infiltration at 6 h posttreatment. Mice with an experimental autoimmune encephalomyelitis (EAE) clinical score of 2.5–3 were randomized into two groups. Mice in one group received an intraperitoneal injection of 200 ng $1,25\text{-}(OH)_2D_3$ in 0.1 ml oil, whereas mice in the other group received oil only as the placebo. The spinal cord samples were collected 6 h later. The spinal cords were fixed in paraformaldehyde, embedded in paraffin, sectioned longitudinally and stained with hematoxylin and eosin. Slides were examined using a Zeiss Axioskop microscope equipped with a Plan-Neofluar 20×/0.5 objective. Bright-field images were acquired with AxioVision 3.0 software controlling an Axiocam digital camera. The nuclei of infiltrating, inflammatory cells appear as dark blue spots. Images are representative of 3 mice per group.

mune response. Two proteosome subunits and cathepsins B, C, D, L, and S increased with EAE disease. The proteosome and cathepsins participate in antigen processing (59) and presentation (32), respectively. Several major histocompatibility (MHC) class I genes associated with antigen presentation, and genes involved with MHC class II molecule assembly, increased significantly in EAE. There were also gene expression increases consistent with leukocyte infiltration and activation. The CD45 (72), CD52 (19), CD72, and lymphocyte antigen 6 (78) genes expressed on activated T and B cells, and the CD86 (15), CD9 (55), S100A8 (33, 79), and toll-like receptor 6 (71) genes expressed on monocytes and dendritic cells, all increased in EAE disease compared with normal mice. A real-time PCR analysis confirmed the trend toward increased CD45 gene expression in EAE disease, but this trend did not reach significance ($P = 0.07$; data not shown). These gene expression changes were consistent with the clinical and histopathological data indicating that EAE disease induction led to a strong inflammatory autoimmune response in the CNS.

A hallmark of MS pathology is degradation of the myelin sheath due to oligodendrocyte injury or loss (4). Since the complement system may inflict damage on oligodendrocytes in the presence of antibodies to myelin basic protein (74), it is noteworthy that several genes encoding complement-associated molecules increased. In addition, two genes encoding myelin sheath proteins decreased (Supplemental Table S1), suggesting that the oligodendrocytes were producing less proteolipid protein (−5.3-fold, $P = 0.009$) and myelin oligodendrocyte glycoprotein (−2.2-fold, $P = 0.009$) due to cell injury or loss, consistent with the EAE histopathology analysis (Fig. 1).

$1,25\text{-}(OH)_2D_3$-induced changes in gene expression. We next compared the gene expression patterns in the CNS of the $1,25\text{-}(OH)_2D_3$-treated and placebo-treated mice with EAE (Table 3). In this acute EAE disease model, primarily $CD4^+$ T cells and $CD11b^+$ macrophages infiltrate the CNS (50). The CD4 and CD11b gene expression did not differ between the hormone-treated and placebo-treated mice with EAE, indicating that the two groups did not differ with respect to $CD4^+$ T cell and $CD11b^+$ macrophage abundance in the spinal cords, consistent with the clinical and histopathological data (Table 1) and the flow cytometric data (not shown). In addition, no significant differences were observed for the inflammatory leukocyte surface markers discussed above. Therefore, the gene expression differences discussed below are attributable to hormone-induced changes and not to differences in the inflammatory cell populations in the spinal cord samples.

Three known vitamin D-responsive genes were among the genes whose expression changed in the $1,25\text{-}(OH)_2D_3$-treated mice compared with the placebo-treated mice, confirming that the $1,25\text{-}(OH)_2D_3$ reached the CNS within 6 h. The genes encoding fatty acid binding protein 3 (16) and calcium-activated neutral protease calpain-2 (62) increased (Table 3), whereas the tubulin-α3 gene (52) decreased (Supplemental Table S2).

Comparing the gene expression patterns of the $1,25\text{-}(OH)_2D_3$-treated and placebo-treated mice with EAE, we found that the $1,25\text{-}(OH)_2D_3$ treatment appeared to alter the expression patterns of 0.7% of the genes analyzed (84 of 12,488; Student's *t*-test $P \leq 0.05$; Mann-Whitney test $P \leq 0.058$; fold change $\geq |2.0|$). The *q* values for this comparison did not allow a meaningful interpretation, because they led to an estimated false discovery rate near 100%. However, a real-time PCR analysis of 5 genes from this list of 84 genes confirmed 4 of them as true positive discoveries for a false discovery rate of about 20% (Fig. 2). Table 3 presents the results for a selected subset of the vitamin D-responsive genes; Supplemental Table S2 presents all of them.

Several genes whose expression decreased with EAE disease increased to near normal gene expression levels in the $1,25\text{-}(OH)_2D_3$-treated mice. These genes were eukaryotic translation initiation factor 2α kinase 4 (eIF-2α kinase-4), uromodulin, bone morphogenetic protein 15, procollagen type IV, and cadherin-related neuronal receptor I. Conversely, some genes whose expression increased with EAE disease decreased to near normal

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Table 2. *Selected genes whose expression in the CNS was altered by EAE induction*

Gene Family and Gene Name	Placebo-Treated EAE Over Naive			1,25-$(OH)_2D_3$-treated EAE Over Placebo-Treated EAE	
	Fold change	*P* value	*q* value	Fold Change	Percent change
Calcium-related genes					
Cathepsin C	28.0	0.004	0.29	1.8	76%
S100 calcium binding protein A8	10.0	0.001	0.19	1.1	14%
Cathepsin L	5.4	0.002	0.24	−1.1	−6%
Cathepsin S	5.4	0.001	0.23	1.5	46%
S100 calcium binding protein A11	4.5	0.001	0.24	1.3	33%
Cathepsin B	3.1	0.004	0.28	1.1	13%
Cathepsin D	2.0	0.001	0.19	−1.1	−11%
Immune response related genes					
CD45 (leukocyte common antigen)	18.2	0.001	0.19	1.5	45%
CD53 molecule	13.7	0.002	0.24	1.8	80%
Fibronectin 1	10.8	0.001	0.19	1.4	44%
Histocompatibility 2, T region locus 23	9.1	0.002	0.24	2.0	103%
Major histocompatibility complex class I molecule	7.4	0.005	0.30	1.7	74%
Interferon-inducible GTPase	7.4	0.003	0.26	1.8	75%
Complement component 1, q subcomponent, b polypeptide	7.4	0.001	0.19	1.0	2%
Lymphocyte antigen 6 complex	6.9	0.001	0.24	1.6	62%
Histocompatibility 2, D region locus 1	6.0	0.003	0.26	1.7	70%
Lymphocyte antigen 86 (CD86, B7-2)	5.0	0.002	0.24	1.0	2%
Complement component 1, q subcomponent, c polypeptide*	4.9	0.001	0.19	−1.1	−6%
Transforming growth factor, β-induced, 68 kDa*	4.9	0.001	0.19	1.5	53%
Small inducible cytokine A9	4.6	0.001	0.23	1.3	31%
CD9 molecule	4.5	0.003	0.26	1.1	13%
CD52 molecule*	4.5	0.002	0.24	1.8	78%
Glucocorticoid-attenuated response gene-49	4.4	0.001	0.19	1.8	76%
CD72 molecule	4.1	0.002	0.24	1.5	45%
Complement component 1, q subcomponent, a polypeptide	3.7	0.002	0.24	−1.1	−6%
Complement factor H-related protein	3.3	0.001	0.19	−1.4	−27%
Macrophage expressed gene 1	3.3	0.003	0.24	1.3	34%
Complement component 3*	3.2	0.002	0.24	−1.0	−4%
Lymphocyte cytosolic protein 1	2.9	0.001	0.19	1.1	12%
Toll-like receptor 6	2.5	0.004	0.28	1.0	3%
Interferon-stimulated protein (15 kDa)	2.1	0.003	0.26	1.3	35%
Transcription/translation-related genes					
CCAAT/enhancer binding protein (C/EBP), delta	10.4	0.001	0.19	−1.5	−31%
Guanine nucleotide binding protein, β1	2.4	0.003	0.26	1.0	3%
Other genes					
Ferritin light chain 1*	4.1	0.004	0.27	1.5	47%
Phospholipase c neighboring	2.1	0.001	0.19	−1.1	−9%

Results from mice with an EAE clinical score of 2.5–3 ($n = 3$) were compared with the results from the naive mice (n = 3) as described in MATERIALS AND METHODS. Differences were considered significant when $P \leq 0.005$ for Student's *t*-test, $P \leq 0.058$ for the Mann-Whitney test, and the fold difference was ≥2. Thirty-five of the 95 genes that met the criteria for statistical and biological significance are tabulated here. Results from hormone-treated mice with EAE were compared with results from placebo-treated mice with EAE as detailed in MATERIALS AND METHODS. *P* values represent the results of a Student's *t*-test comparing log-transformed signal intensity values, and *q* values are based on the specific gene's *P* value. Percent change was computed as $[(D - P)/P] \times 100$, where P and D were the average signal intensities of the placebo-treated and 1,25-$(OH)_2D_3$-treated groups, respectively. *These genes map to regions known to harbor an EAE susceptibility locus. CNS, central nervous system.

gene expression levels in the 1,25-$(OH)_2D_3$-treated mice. Examples were GFAP, neurotrophic tyrosine kinase receptor type 2 (TrkB), and the retinoic acid receptor-γ (RAR-γ). A real-time PCR analysis confirmed the GFAP gene expression result (Fig. 2). In summary, these data suggest the return of several genes to homeostatic expression levels in the hormone-treated mice.

1,25-$(OH)_2D_3$-induced changes in astrocyte gene expression. It is not known which cells are targeted by the 1,25-$(OH)_2D_3$ during EAE resolution, since immune cells (7, 60), neurons (9, 61, 77), astrocytes (43), oligodendrocytes (6), and microglia (69) express the VDR and are involved in the complex interactions of EAE disease. To gain insight into the target cells for 1,25-$(OH)_2D_3$ and VDR-mediated action in the inflamed CNS, we looked for cell type-specific genes whose expression changed in response to hormone treatment. The astrocyte-specific gene GFAP (8) increased with EAE disease, signaling the formation of reactive astrocytes, and decreased with 1,25-$(OH)_2D_3$ treatment (Table 3), indicating a return of the reactive astrocytes to their homeostatic state. The astrocyte-specific gene calcium/calmodulin-dependent protein kinase II-δ increased with 1,25-$(OH)_2D_3$ treatment (8). These results indicated that astrocytes are early target cells for 1,25-$(OH)_2D_3$-mediated action.

1,25-$(OH)_2D_3$-induced changes in apoptosis-related gene expression. The 1,25-$(OH)_2D_3$ treatment also altered the expression of several apoptosis-related genes. The DNA microarray data (Table 3) and real-time PCR data (Fig. 2) showed significant increases in the transcripts encoding the pro-apoptotic proteins calpain-2 and caspase-8-associated protein 2 in the CNS of the 1,25-$(OH)_2D_3$-treated mice with EAE com-

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Table 3. *Selected genes whose expression in the inflamed CNS was altered by 1,25-$(OH)_2D_3$ treatment*

Gene Family and Gene Name	1,25$(OH)_2D_3$-Treated EAE Over Placebo-Treated EAE[a]		Placebo-Treated EAE Over Naive		1,25$(OH)_2D_3$-Treated EAE Over Naive	
	Fold change	*P* value	Fold change	*P* value	Fold change	*P* value
Vitamin D-responsive genes						
Fatty acid binding protein 3	−2.1	0.030	1.3	0.455	−1.6	0.919
Apoptosis-related genes						
Eukaryotic translation initiation factor 2α kinase 4*	7.2	0.014	−10.0	0.001	−1.4	0.203
Calpain-2	5.9	0.014	−2.9	0.047	2.0	0.033
Caspase-8-associated protein 2	2.6	0.040	−1.5	0.563	1.7	0.286
Mouse IAP-2*	−2.2	0.012	2.0	0.001	−1.1	0.012
Chemokine (C-X-CL12)/SDF-1	−4.5	0.041	1.7	0.468	−2.7	0.025
Immune response-related genes						
Uromodulin	17.4	0.001	−17.0	0.050	1.0	0.717
Glucorticoid-attenuated response gene-16	6.4	0.042	ND		ND	
Pentaxin related gene (PTX3)	2.0	0.022	3.3	0.008	6.7	0.001
CNS-related genes						
Neurotrophic tyrosine kinase receptor B (TrkB)	7.7	0.015	−10.8	0.143	−0.7	0.293
Glutamate receptor subunit (GluR6-2)	2.3	0.029	−1.3	0.513	1.8	0.146
Cadherin-related neuronal receptor 1	2.1	0.039	−2.8	0.008	−1.3	0.102
Calcium/calmodulin-dependent protein kinase IIδ	2.0	0.016	−1.1	0.700	1.7	0.079
Glial fibrillary acidic protein*	−2.0	0.016	3.7	0.001	1.9	0.054
Brain creatine kinase B	−2.1	0.012	1.0	0.856	−2.0	0.015
Retinoic acid receptor-γ	−42.6	0.003	3.8	0.030	11.6	0.497
Other genes						
Procollagen, type IV, α4	2.3	0.041	−2.3	0.023	1.0	0.025

Mice with an EAE clinical score of 2.5–3 were randomized to receive 1,25-$(OH)_2D_3$ in 0.1 ml oil or oil only as the placebo. The RNA samples were collected 6 h later. The signal intensity values were log transformed, and the results for the hormone-treated mice ($n = 3$) were compared with the results from the placebo-treated mice ($n = 3$) as described in MATERIALS AND METHODS. Differences were considered significant when $P \leq 0.05$ for Student's *t*-test, $P \leq 0.058$ for the Mann-Whitney test, and the fold difference was ≥2. Eighteen of the 84 genes that met the criteria for statistical and biological significance are tabulated here. Results from placebo-treated mice with EAE were compared with naive mice ($n = 3$); results from hormone-treated mice with an EAE clinical score of 2.5–3 were compared with the naive mice. *P* values represent the results of a Student's *t*-test comparing log-transformed signal intensity values. *These genes also changed expression significantly with EAE induction. ND, not determined, because of negative values in the reference sample.

pared with the placebo-treated mice. Also, the transcripts for two anti-apoptotic proteins, mouse inhibitor of apoptosis (MIAP-2) and CXCL12 (also termed stromal-derived differentiation factor-1) (70), decreased. These changes in calpain-2, caspase-8-associated protein 2, MIAP-2, and CXCL12 gene expression suggested the hypothesis that the 1,25-$(OH)_2D_3$ might increase the inflammatory cell sensitivity to apoptosis as one contribution to EAE resolution.

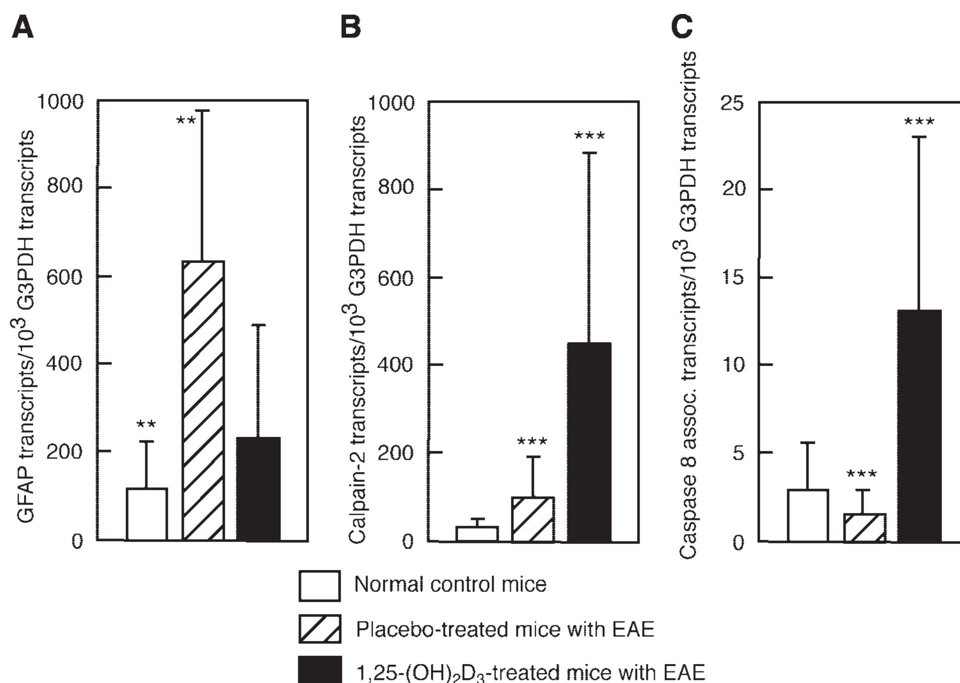

Fig. 2. Real-time PCR analysis confirmed the glial fibrillary acidic protein (GFAP), calpain-2, and caspase-8-associated protein gene expression results. The experimental design is given in the legend to Fig. 1. At 6 h posttreatment, the spinal cord RNA was isolated and reverse transcribed, and quantitative PCR was performed. The results are means and SD; $n = 4$ naive mice/group; $n =$ 6–9 sick, placebo- or 1,25-$(OH)_2D_3$-treated mice/group. $^{**}P < 0.02$ and $^{***}P < 0.01$ (Mann-Whitney test).

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To investigate the apoptosis hypothesis, TUNEL staining was performed on spinal cord sections. The DNA fragmentation that is a hallmark of cells undergoing apoptosis was visualized by detecting the labeled DNA ends immunohistochemically. An increased frequency of apoptotic cells was evident in the spinal cord sections from the hormone-treated mice compared with the placebo-treated mice (Fig. 3). The TUNEL-stained cells were associated with the inflammatory lesions in the meningeal region and did not occur in the white matter or the gray matter of the spinal cord section. This location is consistent with the cells being inflammatory cells responsible for lesional damage, such as neutrophils, macrophages, or $CD4^+$ T cells. Thus the TUNEL results are consistent with the altered gene expression patterns and highlight possible hormone-mediated sensitization of macrophages or $CD4^+$ T cells to apoptotic signals.

A hierarchical RMA cluster analysis was performed (36). The cluster analysis on the EAE-associated genes showed that inflammatory genes in *clusters 1*, *3*, *4*, and *5* increased in expression for the placebo-treated mice with EAE compared with the normal mice (Supplemental Fig. S1 and Supplemental Table S3). The cluster dendrogram for the vitamin D-responsive genes was markedly asymmetrical, reflecting the nonnormal distribution of the data, as was also evident in the *q* value calculation (Supplemental Fig. S2 and Supplemental Table S4). The cluster data showed that there were very few large gene expression changes when the 1,25-$(OH)_2D_3$-treated and placebo-treated mice with EAE were compared. This result indicated that the 6 h time point probably represented initial hormone-induced gene expression changes in the disease resolution pathway.

DISCUSSION

The experiments reported here have provided new insights into how 1,25-$(OH)_2D_3$-mediated activation or repression of vitamin D-responsive genes inhibited EAE, an MS model. It is our hypothesis that 1,25-$(OH)_2D_3$ produced in the CNS performs essential immunoregulatory functions that inhibit the development of MS (30). Administering 1,25-$(OH)_2D_3$ to mice with severe EAE rapidly decreased the inflammatory $CD11b^+$ cell burden in the CNS and reversed the clinical disease signs (50). Here, the cellular targets and mechanisms underlying these effects were investigated through gene expression analysis. The studies were carried out at a time when the injected hormone had reached the CNS but the CNS cell populations had not yet been altered. The results suggested that 2.6% of the transcripts analyzed changed with EAE disease; many of these changes were associated with antigen processing and presentation, leukocyte infiltration and activation, and a pathogenic autoimmune response. In comparison, 0.7% of the transcripts analyzed changed with 1,25-$(OH)_2D_3$ treatment of mice with EAE. Some of the alterations reflected a return to homeostatic levels of transcripts that changed with EAE. Modulations in cell type-specific transcripts indicated that astrocytes were probably direct targets of protective 1,25-$(OH)_2D_3$ actions. In addition, changes in apoptosis-associated genes, together with the TUNEL analysis, indicated that the 1,25-$(OH)_2D_3$ had probably sensitized inflammatory cells to apoptotic signals. The return of particular EAE-associated transcripts to homeostatic levels, the protection of astrocytes, and the sensitization of inflammatory cells to apoptosis would all be consistent with rapid resolution of clinical and histopathological disease (50).

DNA microarrays provide a powerful approach to study hormone-responsive genes and disease mechanisms, but some considerations affect the design and interpretation of microarray studies. Because these studies are costly, the number of treatment groups, time points, and replicates per group is often limited. Our study did not examine the effect of 1,25-$(OH)_2D_3$ on gene expression in the normal spinal cord, because 1,25-$(OH)_2D_3$ does not appear to have major functional roles in the

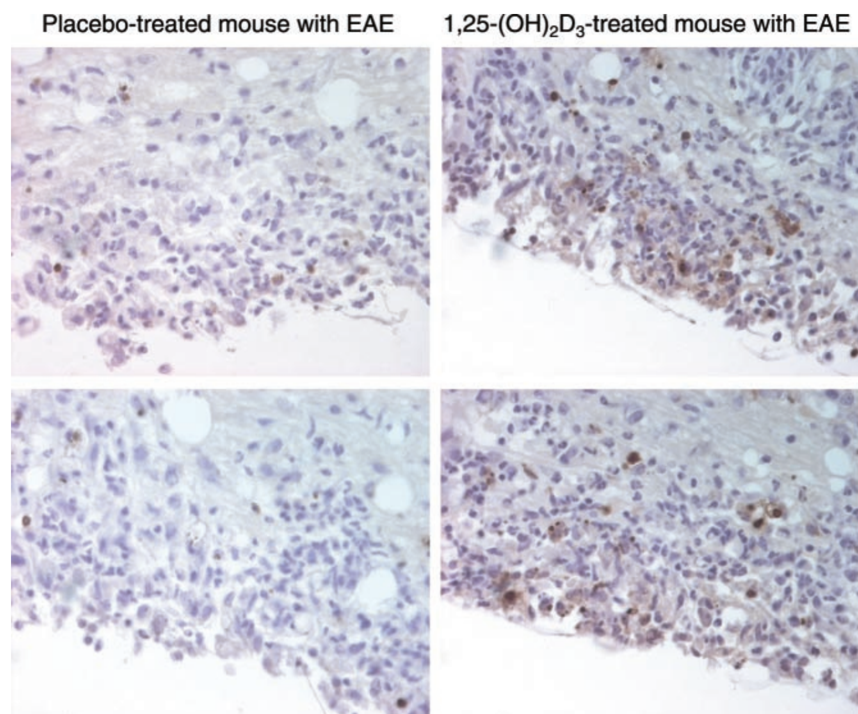

Fig. 3. The 1,25-$(OH)_2D_3$ treatment rapidly increased inflammatory cell apoptosis in the central nervous system (CNS) of mice with severe EAE. The experimental design is given in the legend to Fig. 1. Six hours posttreatment, spinal cords were isolated, fixed, embedded, and sectioned, and in situ TUNEL staining for apoptotic cells was performed. The slides were examined and the images were acquired as described in the legend to Fig. 1. The fragmented DNA of the apoptotic cells appears as darkly stained material. Images are representative of 3 mice per group.

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normal CNS; mice lacking the hormone (17) or the receptor (80) have no neurological phenotype. Our study focused instead on the effect of 1,25-$(OH)_2D_3$ on gene expression in the inflamed spinal cord, where high level 1,25-$(OH)_2D_3$ synthesis and inflammatory cell responsiveness would be expected. Accordingly, it is possible that a fraction of the gene expression changes reported here could be part of the normal CNS response to 1,25-$(OH)_2D_3$, rather than being characteristic of the inflamed CNS response to the hormone. The study was done at one time point, so some very early gene expression changes may have been missed and some of the observed gene expression changes may represent indirect hormone effects. Also, although stringent statistical and fold change criteria were applied, false positives and negatives may be represented in the data. This could be addressed in part by a larger microarray experiment; we had only three replicates per treatment. Finally, the microarrays detect transcript changes that may not be reflected at the protein level. Despite these limitations, the microarray approach is valuable, because gene expression changes may suggest disease resolution mechanisms worthy of further investigation.

The present DNA microarray study examined spinal cord mRNA derived from a heterogeneous mixture of neurons, oligodendrocytes, astrocytes, microglia, and immune cells interacting in a complex micro-environment. Spinal cord mRNA was analyzed to preserve the effects of cell-cell interactions during the hormone treatment. However, gene expression changes in rare cell populations may have been missed. Microarray hybridization analysis can detect gene expression changes in cells that comprise ≥5% of the total cell population (3). The $CD4^+$ T cells comprised <2% of spinal cord cells from mice with EAE (50). This low frequency may explain why IFN-γ, the signature cytokine of pathogenic $CD4^+$ T cells, was not detected in our experiments, or prior EAE (14, 34) or MS (48) studies.

Our results were consistent with the two previous gene expression profiling studies done on spinal cords from mice with acute EAE, yet differences in experimental design and analysis led to some distinctive differentially expressed genes. (14, 34). We confirmed results from Ibrahim et al. (34) for CD53, CD52, complement component 1q β-chain, ferritin light chain 1, complement component 3, and Ia-associated invariant chain. We also confirmed results from Carmody et al. (14) for MHC class I molecules (e.g., H-2 D region locus 1 and others), chemokine receptors (e.g., chemokine C-C receptor 1), cytokines (e.g., small inducible cytokine A9), IFN-inducible transcripts (e.g., IFN-inducible GTPase), cathepsins (e.g., cathepsin C), proteosome subunits, leukocyte surface markers (e.g., CD53 and toll-like receptor 6), S100 calcium binding protein A11 (also known as calgizzarin), and others. Importantly, our microarray study also suggested an increase in CD45 transcripts, consistent with inflammatory cell infiltration. CD45 is a plasma membrane tyrosine phosphatase that regulates T and B lymphocyte receptor signaling (72). A CD45 point mutation has been implicated in the development of MS in some families (37). The three EAE studies are consistent with a gene expression analysis of MS lesions compared with normal control samples (48). Noteworthy in the MS study were increases in MHC class I and II, complement 1 and 3, calgizzarin, and markers of T cells, B cells, and macrophages. In summary, there was a remarkable consistency in the three studies of EAE and the study of MS in that all of the studies detected genes involved in antigen processing and presentation, leukocyte infiltration and activation, and other immune-related molecules.

When 1,25-$(OH)_2D_3$ was administered to mice with severe acute EAE, the leukocyte marker genes did not change, consistent with the clinical, histopathological, and biological data indicating that the hormone had not altered the leukocyte populations. Several genes that changed with EAE returned to near normal expression levels. Examples are GFAP, eIF-2α kinase 4, MIAP-2, TrkB, cadherin-related neuronal receptor I, and RAR-γ. It was surprising that genes associated with antigen processing and presentation, leukocyte infiltration and activation, and immune-mediated pathology were not among the vitamin D-responsive genes. In particular, the placebo and 1,25-$(OH)_2D_3$-treated groups did not differ significantly in their IL-1, IL-2, IL-4, IL-12, TNF-α, IFN-γ, or iNOS gene expression, despite prior in vitro studies reporting that 1,25-$(OH)_2D_3$ regulated these genes (reviewed in Ref. 30). It is possible that these genes are indirectly regulated by 1,25-$(OH)_2D_3$ in vivo, or that the 1,25-$(OH)_2D_3$ level achieved in the CNS in the present experiments was lower than the level used in the in vitro studies, or that these were false-negative data points in our study due to the small sample size or large within group variance.

Our data indicate that the 1,25-$(OH)_2D_3$ rapidly affected reactive astrocytes. Astrocytes perform both pro-inflammatory (20) and anti-inflammatory (26, 58) functions, contributing to homeostasis in the CNS (20). The 1,25-$(OH)_2D_3$ decreased GFAP transcripts. GFAP is an astrocyte-specific intermediate filament protein that increases significantly in the reactive astrocytosis of acute MS (23, 65). On the other hand, the 1,25-$(OH)_2D_3$ increased calcium/calmodulin-dependent protein kinase II-δ transcripts. This kinase functions as a Ca^{2+} spike frequency detector (73), phosphorylating PEA-15 (phosphoprotein enriched in astrocytes) and preventing astrocyte apoptosis (42). Together, these data suggest that the 1,25-$(OH)_2D_3$ likely decreased the astrocyte immune activation state and enhanced astrocyte survival.

Our microarray and PCR data showed a significant increase in transcripts encoding calpain-2 and caspase-8-associated protein 2 in the 1,25-$(OH)_2D_3$-treated animals. The experimental design does not allow us to determine which cells are undergoing apoptosis, although a vast literature exists linking calpain-2 and caspase-8-associated protein 2 to T cell apoptosis. Calpain-2 initiates an apoptosis program in response to an increase in cytosolic calcium (38), and T cell receptor (TCR) engagement causes an increase in cytosolic calcium (40, 66). Moreover, mature, activated T lymphocytes are known to have a calcium- and calpain-2-dependent apoptosis program that is triggered through the TCR (64). Thus, if the 1,25-$(OH)_2D_3$ increased the calpain-2 in the T cells, then TCR engagement might induce cell death rather than stimulation. Similarly, caspase-8-associated protein 2 (also known as FLASH, for "FLICE-associated huge protein") is essential for caspase-8 activation (35), and caspase-8 activation is essential for CD95 (FAS) death receptor-mediated apoptosis in T lymphocytes (63). Thus, if the 1,25-$(OH)_2D_3$ increased the caspase-8-associated protein 2 in the T cells, then these cells might have greater sensitivity to CD95 (FAS)-mediated apoptosis

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triggered by astrocytes expressing CD95L (18, 41, 58). Since there are few links between calpain-2, caspase-8-associated protein 2, apoptosis, and $CD11b^+$ cells, we hypothesize the 1,25-$(OH)_2D_3$ sensitizes T cells to both TCR-mediated and CD95 (FAS)-mediated apoptosis through upregulation of calpain-2 and caspase-8-associated protein 2. Current experiments are examining this possibility. Apoptotic elimination of encephalitogenic $CD4^+$ T cells is a well-documented early step in recovery from EAE and is essential for preventing MS (26, 58).

In summary, this study of 1,25-$(OH)_2D_3$-induced gene expression changes in the EAE treatment model has provided important new insights into how 1,25-$(OH)_2D_3$ may regulate CNS inflammation (30). If there is sufficient sunlight and vitamin D_3, then the microglia (25, 81), neurons (25, 81), and IFN-γ-activated macrophages could produce 1,25-$(OH)_2D_3$ locally in the CNS. The locally produced 1,25-$(OH)_2D_3$ could then act via a paracrine pathway to regulate nearby cells. Our results indicated that the hormone probably exerted protective effects on astrocytes and, at the same time, may have increased the sensitivity of encephalitogenic $CD4^+$ T cells to TCR- and death receptor-mediated apoptotic signals. It is our hope that increasing our understanding of the complex interactions between the vitamin D endocrine system, the immune system, and the CNS will bring us closer to a time when vitamin D-based strategies can be devised to prevent and treat MS.

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