Expression of TWEAK and Its Receptor Fn14 in the Multiple Sclerosis Brain: Implications for Inflammatory Tissue Injury

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Abstract

The expression patterns of tumor necrosis factor-like weak inducer of apoptosis (TWEAK), a pleiotropic cytokine with proinflammatory and cell death-inducing activities, and its receptor, fibroblast growth factor-inducible 14 (Fn14), were examined in postmortem brain tissue samples from patients with multiple sclerosis (MS) and controls. Immunohistochemical analysis and real-time reverse transcription-polymerase chain reaction demonstrated that both TWEAK and Fn14 were upregulated in the MS compared with control unaffected brain samples. Perivascular and meningeal macrophages and astrocytes and microglia associated with lesions were identified as the main sources of TWEAK in the MS brains. The highest frequency of TWEAK+ cells was found at edges of chronic active white matter lesions and in subpial cortical lesions in MS cases with abundant meningeal inflammation and ectopic B-cell follicles. Neurons and reactive astrocytes expressing Fn14 were mainly localized in the cerebral cortex in highly infiltrated MS brains. Numerous TWEAK-expressing microglia were associated with the extensive loss of myelin and astrocytosis, neuronal damage, and vascular abnormalities in subpial cortical lesions; this suggests that TWEAK could synergize with other cytotoxic factors diffusing from the inflamed meninges to promote cortical injury. Taken together, these findings indicate that the TWEAK/Fn14 pathway contributes to inflammation and tissue injury and is, therefore, a potential therapeutic target in MS.

Key Words: Cortical lesions, Cytokines, Inflammation, Microglia, Multiple sclerosis.

INTRODUCTION

The tumor necrosis factor (TNF)-like weak inducer of apoptosis (TWEAK) is a member of the TNF superfamily; it is first synthesized as a Type II transmembrane protein but is usually processed by furin proteases into a secreted soluble cytokine (1). TWEAK acts through its cognate receptor, the Type I transmembrane protein fibroblast growth factor–inducible 14 (Fn14), which seems to signal via recruitment of several different TNF receptor-associated factors, resulting in activation of nuclear factor-κB and mitogen-activated protein kinase pathways (2–4). Like other members of the TNF superfamily, TWEAK can trigger pleiotropic biologic activities, including induction of cell death (1, 5–8), proliferation (9–11), angiogenesis (12–15), and inflammation (1, 16–19) in a context-dependent manner in different cell types.

TWEAK messenger RNA (mRNA) is constitutively expressed in a variety of tissues and cell types and is strongly upregulated locally in tissues with acute injury, inflammatory disease, and cancer (19). On the other hand, Fn14 is often barely detectable in normal healthy tissues but can increase dramatically on various resident cell types of endothelial, epithelial, and mesenchymal origin in settings of tissue injury and disease (19). Whereas Fn14 is highly inducible by growth factors such as fibroblast growth factor, platelet-derived growth factor, and vascular endothelial growth factor (14, 19–21), the transcriptional regulation of TWEAK expression is less well understood. To date, only interferon-γ (IFN-γ) has been identified as a potent inducer of TWEAK in monocytes (22).

Increasing evidence indicates that activation of the TWEAK/Fn14 pathway may play an important role in certain pathological conditions of the central nervous system (CNS) (23). Increased expression of TWEAK and Fn14 mRNA has been demonstrated in the CNS of animal models of multiple sclerosis (MS), experimental autoimmune encephalomyelitis (9, 24), and stroke (25, 26). Moreover, inhibition of the TWEAK/Fn14 pathway had a beneficial effect in both disease models (24, 26). TWEAK has been implicated in CNS inflammation and ischemic damage through its ability to stimulate the production of cytokines and chemokines (9, 24, 27), increase blood-brain barrier permeability (28), and induce neurodegeneration (25). Studies in rodents indicate that TWEAK is constitutively expressed or can be upregulated in astrocytes, microglia, and neurons in vitro and/or
in vivo (9, 25, 26), whereas Fn14 expression has been detected mainly in neurons and to a lesser extent in astrocytes (9, 26, 29). The expression patterns of TWEAK and Fn14 in the normal human brain and their regulation in inflammatory and neurodegenerative diseases, however, have not yet been characterized.

Multiple sclerosis is a chronic inflammatory disease of the CNS that causes progressive neurological disability (30). Pathologically, MS is characterized by the presence of focal demyelinated lesions in the white and gray matter and by preferential accumulation of blood-borne inflammatory cells around white matter blood vessels and in the meninges (31). Blood-brain barrier disruption with edema occurs during acute inflammation, as assessed by gadolinium-enhanced magnetic resonance imaging (32). Within MS lesions, reactive astrocytosis, microglia activation, axonal damage, and neuronal loss are also observed (31). In the meningeal compartment, organization of the inflammatory infiltrates into structures that resemble B-cell follicles has been observed in a subset of MS patients with secondary progressive disease; these structures are associated with extensive cortical damage (33, 34).

Multiple sclerosis lesions have traditionally been thought to be caused by an autoimmune attack on myelin (35). Recently, work from our group has challenged this pathogenetic model by demonstrating an abnormal accumulation of B cells and plasma cells infected with Epstein-Barr virus (EBV) in MS lesions and meninges, with the highest frequency observed in intrameningeal B-cell follicles (36). The latter were also identified as major sites of EBV reactivation. In the same study, we provided evidence that CD8 T cells infiltrating EBV deposits in the MS brain display cytotoxic activity toward virus-infected B cells/plasma cells (36). These findings led us to propose that MS lesions may instead result from a persistent and dysregulated EBV infection in the CNS with subsequent immunopathologic injury. An antiviral host immune response may induce local production of TNF family molecules (37), which in turn contribute to amplify inflammation and tissue damage (38). In this study, therefore, we evaluated the expression levels and cellular localizations of TWEAK and its receptor Fn14 in postmortem MS brain specimens. We paid particular attention to TWEAK distribution in highly infiltrated meninges containing ectopic follicles and in the adjacent gray matter, in which extensive demyelination and marked neuronal loss were recently reported (34).

**MATERIALS AND METHODS**

**Postmortem Brain Tissue Specimens**

Immunohistochemical analyses were performed on postmortem brain tissue from 2 patients without neurological disease, 9 cases with secondary progressive MS (SPMS), and 1 case with primary progressive MS. Control and MS brain tissue specimens were provided by the United Kingdom Multiple Sclerosis Tissue Bank at Imperial College London. Confirmation of MS diagnoses was provided by Drs F. Roncaroli and R. Nicholas. The postmortem delays ranged from 7 to 24 hours (median time, 16.5 hours). The CNS tissues were fixed in 4% paraformaldehyde for 7 days, cryoprotected in sucrose, frozen by immersion in isopentane, precooled on a bed of dry ice (−55°C), and stored at −80°C until use. This study was approved by the ethics committee of the Istituto Superiore di Sanità, Rome, Italy. For the MS cases, 13 tissue blocks (2 × 2 cm) from the cerebral hemispheres were analyzed and classified by histopathologic methods to identify areas of inflammation and demyelination, according to De Groot et al (39) and Peterson et al (40). Both gray and white matter lesions and the adjacent areas were analyzed. The total number of lesions analyzed was 23 in the white matter, of which 1 was classified as active, 14 as chronic active, and 8 as chronic inactive, and 25 in the gray matter of which 21 were subpial (or Type III) lesions (2 active, 13 chronic active, and 6 inactive) and 4 were leukocortical (or Type I) (1 active and 3 chronic active), according to Peterson et al (40).

For gene expression studies, snap-frozen brain tissue samples from 9 control and 16 SPMS cases were used. This series included 7 of the MS cases that were used for immunohistochemical analyses. A fine scalpel blade was used to delineate and excise areas of gray matter in whole snap-frozen tissue blocks from the precentral gyrus (motor cortex) of control and MS cases; the cut was made within Layer VI of the cerebral cortex to avoid white matter contamination. For MS cases, tissue blocks containing gray matter lesions were selected using immunostaining for myelin oligodendrocyte glycoprotein (MOG) on frozen sections. Serial tissue sections (20- to 50-μm thick) were then carefully cut on a Leica cryostat (Leica Microsystems UK, Milton Keynes, United Kingdom). Fifty- to 150-mg tissue samples were collected for each case.

**Immunohistochemistry**

Air-dried acetone-fixed 10-μm-thick cryosections were immunostained with the following antibodies (Abs): anti-TWEAK monoclonal Ab (mAb) (clone P2D10; Biogen Idec, Cambridge, MA), and anti-Fn14 mAb (clone ITEM4; Acris Antibodies, Herford, Germany), anti-Iba-1 (ionized calcium binding adapter molecule-1) rabbit polyclonal Ab (a kind gift of Dr Y. Imai, Tokyo, Japan) for macrophages/microglia; anti-CD20 for B cells (Immunotech, Marseille, France); anti-immunoglobulin A, M, G polyclonal Ab (Dako, Carpinteria, CA) for plasma cells, anti-HLA-DR mAb (CR3/43, Dako), anti-MOG mAb (Serotec, Oxford, United Kingdom) for myelin staining, anti-glial fibrillary acidic protein rabbit polyclonal antibody (Dako) and mAb (BioGenex, San Ramon, CA) for astrocytes, anti-cleaved caspase-3 rabbit polyclonal Ab (Cell Signaling Technology, Boston, MA) to stain apoptotic cells; anti-C3D1 mAb for endothelial cells (Dako), anti-laminin rabbit polyclonal Ab for the basal membrane (Dako), and anti-neurofilament (200 kd) rabbit polyclonal antibody for neurons (DBA, Segrane, Milan, Italy). For single immunostaining, postfixed sections were subjected to the antigen retrieval procedure by microwave irradiation in citrate buffer (10 mM, pH 6.0). Sections were incubated for 20 minutes with 0.1% hydrogen peroxide in PBS to eliminate endogenous peroxidase activity, for 1 hour with 10% of

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normal sera (Jackson ImmunoResearch, Cambridgeshire, United Kingdom), and overnight at 4°C with the primary Abs diluted in PBS containing 1% bovine serum albumin. The binding of biotinylated secondary Abs (rabbit anti-mouse immunoglobulin G and donkey anti-rabbit immunoglobulin, from Jackson ImmunoResearch Laboratories) was visualized with the avidin-biotin horseradish peroxidase complex technique (ABC Vectastain Elite kit; Vector Laboratories, Burlingame, CA) and 3,3′-diaminobenzidine (Sigma, St Louis, MO) as substrate. All sections were counterstained with hematoxylin, sealed with Canadian Balsam, viewed and photographed with an Axioshot Zeiss microscope equipped with an Axiocam digital camera, using the Axiovision 4 AC software. Negative controls included the use of immunoglobulin G isotype controls or preimmune sera or omission of the primary antibodies.

For single indirect immunofluorescence staining with anti-CD20 mAb, a fluorescein-conjugated goat anti-mouse secondary antibody was used. For double indirect immunofluorescence staining, sections were incubated overnight with a mixture of primary antibodies in 20% normal serum. Sections were then washed, treated with rhodamine-conjugated goat anti-mouse and fluorescein-conjugated goat anti-rabbit Abs or with fluorescein-conjugated donkey anti-mouse and Cy3-conjugated donkey anti-rabbit Abs for 1 hour at room temperature, washed in PBS, and sealed in Vectashield (Vector Laboratories). Fluoro-Jade B histofluorescence staining, a method to selectively identify degenerating neurons, was performed according to the manufacturer’s instructions (Chemicon, Temecula, CA). Images were analyzed and acquired with a laser scanning confocal microscope (LSM 5 Pascal, Carl Zeiss).

Quantification of TWEAK, Fluoro-Jade, and Cleaved Caspase 3+ Cells in the Cerebral Cortex

To evaluate the frequency of TWEAK+ cells in control and MS brains and to correlate TWEAK expression with cell damage in cortical lesions, quantification of cells immunoreactive for TWEAK, Fluoro-Jade, and cleaved caspase-3 was performed at 500× magnification in 15 to 20 different fields of 2 sections/tissue block for each marker. Fluoro-Jade B cells were counted throughout the cerebral cortex, whereas cleaved caspase-3+ cells and TWEAK+ cells were counted in the first 3 layers of the cerebral cortex (molecular layer, external granule layer, and external pyramidal layer). Cell density was expressed as the mean ± SEM of positive cells/mm². Values of p calculated by Student t-test were considered significant when equal to or less than 0.05.

Real-Time Reverse Transcription–Polymerase Chain Reaction Assay

Total RNA was extracted from brain sections using the RNeasy Lipid Tissue Midi Kit (Qiagen, Hilden, Germany). One microgram of purified RNA was reverse transcribed using the QuantiTect kit (Qiagen) according to the manufacturer’s instructions. Each complementary DNA sample was amplified in duplicate for TWEAK and FN14 genes and for GAPDH as a housekeeping gene. The following probe assays from Qiagen were used: for TWEAK, Hs-TNFSF12-1-SG QuantiTect Primer Assay (QT00202545); for FN14, Hs-TNFRSF12A-1-SG QuantiTect Primer Assay (QT00221179); for GAPDH, Hs-GAPDH-2-SG QuantiTect Primer Assay (QT01192646).

Amplifications were performed using the QuantiTect SYBR Green PCR kit (Qiagen) on a Stratagene Mx3000P cycler (Agilent Technologies, Santa Clara, CA).

Relative amounts of the target genes were calculated using the comparative Ct (threshold cycle number at a cross-point between amplification plot and threshold) method and normalized to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control. The ΔΔCt method was applied to quantify TWEAK and FN14 mRNA expression using a pool of samples from all the control cases as calibrator. Expression levels of TWEAK and Fn14 in the cortical gray matter of control and MS cases were compared using the Mann–Whitney U test; values of p < 0.05 were considered significant.

RESULTS

Immunohistochemical analyses were performed in cerebral sections from 9 cases with SPMS and 1 case with primary progressive MS that were included in a previously characterized series (34) and 2 controls. Prominent brain inflammation, intrameningeal B-cell follicles, and enhanced cortical damage were detected in 5 of the 10 MS cases examined.

TWEAK Expression in Control and MS Brain Samples

White Matter

TWEAK was generally undetectable by immunohistochemistry in the normal human brain white matter samples (Fig. 1A). In the 5 MS cases with significant brain inflammation (all of which were SPMS cases), but not in the less infiltrated cases, strong TWEAK immunoreactivity was observed in reactive astrocytes along the border of chronic active demyelinated lesions (Figs. 1B, C) and in cells with morphological features of macrophages within perivascular inflammatory cuffs (Fig. 1D). Large cells located in the parenchyma at the edge of chronic active lesions that were most likely foamy macrophages were also occasionally weakly immunoreactive for TWEAK (Fig. 1D, arrows). By double immunofluorescence staining with anti-TWEAK and anti-Iba-1 Abs, all TWEAK+ cells in the perivascular cuffs were identified as macrophages; about half of the perivascular macrophages expressed TWEAK (Fig. 1E). No Iba-1+/TWEAK+ cells could be visualized by immunofluorescence within the parenchyma of chronic active lesions (Fig. 1E). A high frequency of faintly stained TWEAK+ activated microglia were, however, detected in the only active white matter lesion analyzed in this study (Fig. 2). No TWEAK+ cells were detected in inactive lesions or normal-appearing white matter (data not shown).

Cerebral Cortex

TWEAK was generally undetectable in the normal human cerebral cortex samples (Fig. 3A). Some faintly...
stained glial cells, possibly astrocytes, were occasionally observed only in the cortex (Fig. 3A). With the exception of some areas bordering the white matter (leucocortical or Type I lesions), the cortical gray matter of MS brains showed a variable extent of demyelination but was not infiltrated by blood-derived inflammatory cells, consistent with previous reports (40, 41). In contrast to white matter lesions, strong TWEAK immunoreactivity was detected in the cerebral cortex of all 10 MS cases examined, particularly in lesions located beneath the pial membrane (subpial or Type III cortical lesions; Figs. 3B, C). The TWEAK+ cells were mainly localized in active and chronic active gray matter lesions and were nearly absent in inactive lesions. Within cortical lesions, most TWEAK+ cells had morphological appearances

**FIGURE 1.** Expression and cellular localization of TWEAK in the white matter of control and multiple sclerosis cases. (A) White matter of a control case is largely negative. (B–E) Immunostaining for TWEAK in chronic active white matter lesions from 2 different secondary progressive multiple sclerosis cases. There is strong immunoreactivity for TWEAK in cells with morphological features of astrocytes at the lesion borders (B, C) and in sparse leukocytes in the perivascular inflammatory cell infiltrates (D). A faint signal for TWEAK is also observed in intraparenchymal cells that resemble foamy macrophages (arrows). (E) Double immunofluorescence staining for the macrophage marker Iba-1 (green) and TWEAK (red) shows that TWEAK staining is restricted to perivascular macrophages; TWEAK is not detected in the Iba1+ ramified microglia surrounding the inflamed blood vessel. Original magnification: (A) 250×; (B, D) 500×; (C) 1,000×. Scale bar = (E) 50 μm.

**FIGURE 2.** Expression of TWEAK in microglia in an active white matter lesion. (A) The acute inflammatory lesion is characterized by the presence of a central inflamed venule and largely preserved myelin (myelin oligodendrocyte glycoprotein [MOG] staining, inset). The TWEAK antibody strongly stains perivascular cells, whereas numerous faintly immunoreactive TWEAK+ cells are scattered throughout the lesion. (B) At higher magnification (and inset therein), the intraparenchymal TWEAK+ cells have an enlarged cell body and short thick processes consistent with activated microglia. (C) There is marked microglial activation (double immunofluorescence staining for Iba-1 and major histocompatibility complex [MHC] class II) in this lesion. Original magnification: ([A] and inset) 250×; ([B] and inset) 500×. Scale bar = (C) 50 μm.
consistent with activated microglia (insets in Figs. 3B, C). The TWEAK+ cells were more numerous in subpial lesions of the 5 MS cases with ectopic B-cell follicles than in those without (compare Fig. 3B and Fig. 4A with Fig. 3C; Table); this is consistent with the previously reported difference in the number and extension of active lesions and the frequency of activated microglia between the 2 subsets (34). Microglial activation in the same areas was confirmed by double immunofluorescence staining for major histocompatibility complex (MHC) class II and Iba-1 (Fig. 3D). Using double immunostaining for TWEAK and Iba-1, approximately 50% of Iba-1+ microglia in the external cortical layers close to ectopic follicles (asterisks) (Fig. 3E) revealed that MHC class II and TWEAK are expressed in a substantial proportion (nearly half) of Iba-1+ microglia in the external cortical layers close to ectopic follicles (asterisks). In the same SPMS case shown in (D) and (E), numerous astrocytes were TWEAK+ in the transitional zone between the gray matter and the white matter. The upper inset shows TWEAK+ astrocyte-like cells at higher magnification. TWEAK+ endothelial cells in a cortical lesion are shown (lower inset). Original magnification: (A) 250×; (B, C, F) 500×; (inset in A) 1,000×. Scale bar = (D, E) 20 μm.

Meninges

In the inflamed leptomeninges and within ectopic B-cell follicles, TWEAK was expressed by several cells with large irregular cell bodies (Figs. 4A–D). By double immunofluorescence, approximately 80% to 90% of TWEAK+ cells were identified as Iba-1+ macrophages, and nearly all Iba-1+ macrophages were found to express TWEAK (Fig. 4F). The possible expression of TWEAK in B cells and
plasma cells was ruled out by double labeling for TWEAK and CD20 (not shown) or immunoglobulin (Fig. 4E).

**Fn14 Expression in Control and MS Brain Samples**

With the exception of a few Fn14-expressing cells that exhibited an astrocyte morphology and were in contact with blood vessels (Fig. 5A), normal control gray and white matter were largely negative for the TWEAK receptor Fn14. The Fn14 immunoreactivity was detected in the brain parenchyma in MS cases, but not in the meninges (data not shown). In the white matter, Fn14 immunoreactivity seemed largely restricted to reactive astrocytes along the borders of chronic active lesions and in the surrounding normal-appearing white matter (Fig. 5B). The Fn14 immunoreactivity was only occasionally seen on cells with microglial morphology (inset in Fig. 5B). The Fn14 immunoreactivity was also observed on astrocytes in the 5 most inflamed MS brains (Fig. 5C) and on neuronal cell bodies and processes (Figs. 5D, E) both within cortical lesions and in the surrounding normal-appearing gray matter. The Fn14 expression on the surface of the neuronal processes was confirmed by double immunofluorescence staining with anti-Fn14 and anti-neurofilament antibodies (Figs. 5F, G). Despite previous studies that have described inducible Fn14 expression on endothelial cells primarily in in vitro systems (13, 14, 19), no Fn14 was detected on the endothelia of normal and inflamed blood vessels in the brain parenchyma and meninges of any of the MS cases.

**TABLE.** Quantification of TWEAK+ Cells, Dying Neurons, and Apoptotic Cells in the Cerebral Cortex of Secondary Progressive Multiple Sclerosis Cases With Different Degrees of Meningeal Inflammation

<table>
<thead>
<tr>
<th></th>
<th>No. TWEAK+ Cells/mm²</th>
<th>No. Fluoro-Jade+ Cells/mm²</th>
<th>No. Cleaved Caspase-3+ Cells/mm²</th>
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<tr>
<td>MS cases with ectopic B-cell follicles (n = 5)</td>
<td>260 ± 20*</td>
<td>0.15 ± 0.01†</td>
<td>28 ± 9*</td>
</tr>
<tr>
<td>MS cases without ectopic B-cell follicles (n = 5)</td>
<td>119 ± 33</td>
<td>0.02 ± 0.01</td>
<td>3.9 ± 1.8</td>
</tr>
<tr>
<td>Control (n = 2)</td>
<td>0</td>
<td>0.005</td>
<td>0</td>
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Quantification of immunopositive cells was performed at 500× magnification in 15 to 20 different fields of 2 sections/tissue block for each marker. Cell density is expressed as the mean ± SEM of positive cells/mm². The TWEAK+ cells and cleaved caspase-3+ cells were counted in bright field in the molecular, external granule, and external pyramidal layers of the cerebral cortex. Fluoro-Jade+ cells were counted throughout the cerebral cortex. Statistically significant differences in the density of stained cell types in secondary progressive multiple sclerosis cases with follicles versus those without follicles were calculated using the Student t-test. *p < 0.05. †p < 0.005. MS, multiple sclerosis.
MS cases analyzed. Whether this reflects an intrinsic inability of cerebrovascular endothelial cells to upregulate Fn14 or whether it is caused by the detection limits of immunohistochemical techniques remains to be clarified. Using immunocytochemistry and flow cytometry analysis, we found that the Fn14 expression level on human umbilical vein endothelial cells is significantly lower than that on cultured primary human astrocytes (T. S. Zheng, unpublished data).

**TWEAK and Fn14 Gene Expressions in the Cortex of Control and MS Cases**

To corroborate the immunohistochemical findings, we also measured the expression levels of TWEAK and Fn14 mRNA in cortical lesion samples from 9 control cases and 16 SPMS cases (8 with follicles and 8 without). Because TWEAK immunoreactivity was more consistently found in cortical gray matter lesions than in white matter lesions, gene expression analysis was restricted to the cerebral cortex. Expression of TWEAK and Fn14 transcripts was observed in all 9 control samples analyzed (data not shown), with moderate variability in relative expression levels (median value, 0.9 and range, 0.7–1.7 for TWEAK; median value, 1.1 and range, 0.3–3.9 for Fn14; Figs. 6A, B). In the 16 MS brain samples, TWEAK and Fn14 mRNA levels varied over a wider range (median value, 1.9 and range, 1.0–7.9 for TWEAK; median value, 2.3 and range, 0.3–17.9 for Fn14; Figs. 6A, B). Despite variability, expression of TWEAK was

![Image](https://example.com/image.png)

**FIGURE 5.** Detection of Fn-14 in the brain of control and MS cases. (A) Immunostaining with anti-Fn14 antibody shows that the normal white matter is largely negative for this molecule; the inset highlights the occasional finding of isolated cells with an astrocytic morphology that are strongly immunoreactive for Fn14. (B–G) Immunostaining performed in highly inflammatory brains from secondary progressive multiple sclerosis (SPMS) cases with ectopic follicles. (B) Immunostaining reveals the presence of several Fn14+ reactive astrocytes at the edge of a white matter lesion; the inset shows one of the rare Fn14+ cells with a microglia-like morphology identified in the same lesion. (C) Some astrocytes are Fn14+ in a subpial lesion close to an ectopic follicle. (D, E) In 2 different SPMS cases with marked meningeal inflammation, Fn14 immunoreactivity is present on some neuronal cell bodies (D) and processes (E). (F, G) Double immunofluorescence staining with anti-Fn14 monoclonal antibody (green) and anti-NF 200 (red) demonstrates Fn14 immunoreactivity on the membrane of neurofilament+ axons. Original magnification: (A, B, D) 500×; (C) and inset in (A) 1,000×. Scale bars = (E) 50 μm; (F) 20 μm.

![Image](https://example.com/image.png)

**FIGURE 6.** TWEAK and Fn14 gene expression in control and MS cortex. TWEAK (A) and Fn14 (B) messenger RNA (mRNA) was quantitated by real-time reverse transcription-polymerase chain reaction in cortical samples dissected from brains of control (n = 9) and multiple sclerosis (MS) cases (n = 16). Data are expressed as mRNA levels normalized to glyceraldehyde-3-phosphate dehydrogenase relative to a sample of pooled control cases used for calibration. Dots represent values for each control or MS case analyzed; bars represent median values. Values of p were calculated with Mann-Whitney U test.
significantly upregulated (Fig. 6A), and there was a trend for increased expression of Fn14 (Fig. 6B) in MS cases compared with controls. There were no significant differences in TWEAK or Fn14 gene expression between MS cases with and without follicles (data not shown).

**Analysis of Cellular Damage in MS Cortical Lesions**

We previously showed that demyelinated lesions in the cerebral cortex of MS cases with prominent meningeal inflammation and ectopic B-cell follicles were more numerous and larger and showed more axonal loss and microglial activation compared with those of MS cases with scarcely infiltrated meninges (34). The subsequent demonstration that highly infiltrated meninges comprise EBV-infected B cells and CD8+ T cells displaying signs of activation and cytotoxicity led us to hypothesize that factors diffusing across the pial membrane could mediate cortical damage directly and/or through activation of microglial inflammatory and cytotoxic functions (36). In view of the high expression of both TWEAK and Fn14 in cortical lesions previously described and the well-documented proapoptotic and proinflammatory properties of TWEAK (19), we evaluated more carefully the tissue damage in the cerebral cortex. In the

![FIGURE 7. Characterization of the cellular damage in the cerebral cortex of multiple sclerosis (MS) patients with and without intrameningeal B-cell follicles. (A) In a secondary progressive MS (SPMS) case with follicles, a large subpial lesion close to an intrameningeal follicle (marked with an asterisk) is revealed by loss of immunoreactivity for myelin oligodendrocyte glycoprotein (MOG). (B) Immunostaining for Iba-1 (green) and MOG (red) in a serial section shows intense microglial activation in the same cortical lesion and the presence of numerous Iba-1+ macrophages inside the ectopic follicle. (C, D) A dramatic loss of astrocytes in the same subpial lesion adjoining the B-cell follicle shown in (A) and (B) is documented by double immunofluorescence staining for glial fibrillary acidic protein (GFAP) (green) and CD20 (red) (C), and for GFAP (green) and MOG (red) (D). (E) In the same MS case, Fluoro-Jade B staining reveals the presence of damaged neurons (arrows) in the normal-appearing gray matter surrounding a subpial lesion. Inset shows higher magnification of a positive neuron. (F) Immunostaining for cleaved caspase-3 reveals the presence of numerous apoptotic cells in Layer I of the cerebral cortex within a subpial lesion close to an ectopic follicle (not included in the selected field). The area within the frame is shown at higher magnification in the inset. (G) Occasional apoptotic neurons are shown in a deeper cortical layer. (H, I) In an SPMS case with scarce meningeal immune infiltrates, both the area of a subpial cortical lesion ([H] immunostaining for MOG) and the astrocytic loss within the same lesion ([I] immunostaining for GFAP in an adjacent section) are less pronounced compared with the SPMS case with intrameningeal follicles (contrast panels [H, I] with panels [A, C, D]). (J) The same lesion and the surrounding normal-appearing gray matter contain no Fluoro-Jade B+ damaged neurons. Original magnification: (A, F) 250×; (inset in [F, G]) 500×; (H) 125×. Scale bar = ([B, E], inset in [E, I, J]) 50 μm. Copyright © 2008 American Association of Neuropathologists, Inc.
subpial gray matter lesions adjacent to ectopic follicles—where microglial activation and TWEAK expression were most prominent—there was profound tissue damage characterized by total loss of myelin (Figs. 7A, B), marked reduction or even complete disappearance of the astrocyte markers glial fibrillary acidic protein (GFAP) (Figs. 7C, D) and S100β (not shown), and the presence of Fluoro-Jade B+ damaged neurons (Fig. 7E; Table). Moreover, immunostaining for cleaved (enzymatically active) caspase-3 revealed the presence of numerous apoptotic cells in Layer I and to a lesser extent Layer II of the cortex (Fig. 7F; Table). Only occasional apoptotic neurons were detected in the deeper cortical layers (Fig. 7G). Interestingly, immunostaining with an anti-laminin Ab, which decorates the perivascular basal lamina, and with the endothelial cell marker CD31 revealed the presence of numerous apoptotic cells in Layer I and to a lesser extent Layer II of the cortex (Fig. 7F; Table). Only occasional apoptotic neurons were detected in the deeper cortical layers (Fig. 7G). Additionally, immunostaining with an anti-laminin Ab, which decorates the perivascular basal lamina, and with the endothelial cell marker CD31 revealed extensive loss of laminin and a marked reduction of the capillary tree in subpial cortical lesions of the 5 MS cases with prominent meningeal inflammation (Figs. 8B, E) as compared with control unaffected brains (Figs. 8A, D). The vascular abnormalities observed in the highly inflamed MS brains were more pronounced in the molecular and external granule layers of the cerebral cortex (Fig. 8), whereas in the deeper cortical layers, immunoreactivity for blood vessel markers was similar to that found in control brain (not shown). Consistent with our previous findings (34), cortical damage was less prominent in terms of demyelinated area (Fig. 7H), astrocyte loss (Fig. 7I), neuronal damage (Table), frequency of apoptotic cells (Table), and vascular abnormalities (Figs. 8C, F) in MS brains with negligible meningeal inflammation and lower TWEAK expression.

**DISCUSSION**

The past few years have witnessed a significant advance in our understanding of how the TWEAK/Fn14 pathway contributes to tissue remodeling under both physiological and pathological conditions (19). Thanks largely to studies in animal models, it is now recognized on the one hand that TWEAK/Fn14 activation may play a beneficial role in promoting tissue regeneration and repair (10, 29, 42), and on the other hand, persistent TWEAK stimulation under robust inflammatory conditions leads to pathological consequences (19). Accordingly, TWEAK blockade proved efficacious in reducing disease severity in murine models of arthritis (18), nephritis (43), MS (9, 24), and stroke (25, 26). In this study, we have explored the potential involvement of TWEAK/Fn14 pathway in human neuroinflammatory diseases by systematically examining the expression patterns of TWEAK and Fn14 in postmortem brain sections from MS cases. The results obtained lend support to the concept that this pathway may contribute to inflammatory and neurodegenerative changes in MS.

First, we found that TWEAK and Fn14 expression levels are considerably upregulated in the brains of MS cases compared with controls. By immunohistochemistry, normal human brain sections showed undetectable to very low levels
of either protein, whereas both TWEAK and Fn14 immunoreactivities were readily detected in MS brain samples. More extensive TWEAK and Fn14 expression was found in MS tissue samples with greater degrees of inflammation and demyelination, thereby directly linking TWEAK/Fn14 activation to MS immunopathology. By real-time reverse transcription–polymerase chain reaction, both TWEAK and Fn14 transcripts were already expressed in the gray matter of control brains and were upregulated in a substantial number of MS cases, although significant differences were found only for TWEAK. The high variability in TWEAK and Fn14 gene expressions found in MS brain samples could be caused by many factors, including the variable extent of lesioned versus nonlesioned areas and the variable degree of inflammation of the lesions from 1 case to another. Taken together, however, the immunohistochemical and gene expression data strongly suggest that the TWEAK/Fn14 pathway is specifically activated in the MS brain.

Second, we identified perivascular and intrameningeal macrophages, as well as astrocytes and microglia, as major sources of TWEAK in the MS brain, although their contribution to TWEAK production differed according to lesion location and degree of inflammation. The highest frequency of TWEAK+ cells was detected inside and around gray matter lesions in the cerebral cortex of MS cases with abundant meningeal inflammation and ectopic B-cell follicles. Within cortical lesions, TWEAK was mainly expressed in activated microglia, whereas TWEAK+ astrocytes predominated in the surrounding nondemyelinated cortex and at the border between the cortex and white matter. Lower numbers of TWEAK+ microglial cells, but no TWEAK+ astrocytes, were detected in cortical lesions of MS cases with little or no meningeal inflammation. These findings suggest that soluble mediators produced in the infiltrated meninges may regulate expression of TWEAK in the adjacent cerebral cortex. Among the numerous mediators released in an inflammatory milieu, IFN-γ has been identified as the major TWEAK-inducing stimulus in cells of the myeloid lineage (22). We have recently shown that the inflamed meninges, particularly ectopic follicles, are enriched in B cells and plasma cells infected with EBV and in CD8+ T cells that show signs of activation, including production of IFN-γ, and cytotoxicity toward the infected cells (36). Thus, it is possible that IFN-γ, together with other cytokines produced as part of an antiviral immune response, may diffuse through the subarachnoid space and across the pial membrane, promoting glial activation and production of proinflammatory mediators, including TWEAK. Consistent with this proposal, numerous TWEAK+ macrophages were also detected in the inflamed meninges and inside ectopic follicles. The finding that a high proportion of microglia in subpial cortical lesions and of macrophages in the inflamed meninges expresses TWEAK (50% and 80%–90%, respectively) indicates that the local cytokine milieu is very effective in inducing TWEAK expression. In view of the current lack of understanding of the regulation of TWEAK expression at the molecular level in vivo, this observation is of great interest. In the lesioned white matter, strong TWEAK immunoreactivity seemed to be confined to astrocytes at the borders of chronic active lesions and to perivascular macrophages; weak immunoreactivity for TWEAK was detected in a substantial number of intraparenchymal microglia-like cells only in an active MS lesion. Because EBV-infected B cells and activated CD8+ T cells displaying cytotoxic activity and IFN-γ production mainly localize in the perivascular cuffs of active and chronic active white matter lesions (36), factors diffusing from the perivascular space are likely to play a major role in determining alterations in the surrounding parenchyma. The low level of TWEAK production by microglia in white matter lesions suggests that TWEAK-inducing stimuli originating at these sites might be weaker than those diffusing from the inflamed meninges into the gray matter. It is also likely that the pial membrane and the underlying glial limitans are more permeable to soluble factors than the perivascular basal lamina and astrocytic processes that form the blood-brain barrier. Another possibility is that macrophages engaged in myelin phagocytosis in white matter lesions adopt an anti-inflammatory phenotype and are refractory to stimuli upregulating certain proinflammatory mediators, including TNF family members (44). Because activated microglia in cortical lesions do not transform into foamy macrophages, the maintenance of a proinflammatory phenotype, as indicated by abundant TWEAK production, for extended periods could have a major impact on neuronal injury and degenerative processes.

In previous studies, the expression of another member of the TNF superfamily (i.e. TNF itself) was found to be restricted predominantly to perivascular macrophages and intraparenchymal cells with macrophage or microglial morphology in white matter MS lesions (45, 46). When TWEAK and TNF expression patterns were compared in the same MS brain samples, we observed that the frequency of TNF-producing cells was much lower than that of TWEAK-producing cells, mainly because of the absence of TNF+ astrocytes and considerably fewer TNF+ microglia. Although TNF+ cells were commonly found in the perivascular cuffs and sparse meningeal inflammatory infiltrates, intraparenchymal TNF-producing cells with a microglial or macrophage morphology were rarely observed in white and gray matter lesions of the most inflamed MS brains, and none was present inside ectopic follicles (R. Magliozzi, unpublished data). These findings suggest a different type of regulation of the 2 molecules in vivo.

Third, we found that Fn14 immunoreactivity was mainly localized in neurons and reactive astrocytes in the cerebral cortex of highly infiltrated MS brains. Conversely, Fn14 was undetectable in cerebrovascular endothelia and microglia in both the lesioned white and gray matter. The characterization of the cell types that express Fn14 in the context of MS provides insight into the potential molecular mechanisms by which TWEAK may mediate brain injury. Based on previously reported activities of TWEAK (19, 23), the identification of reactive astrocytes and neurons in the brain parenchyma as the major TWEAK-responsive cells suggests that the underlying pathogenic contribution of TWEAK in MS involves both amplification of inflammation and direct tissue damage. It has been well documented that TWEAK can stimulate cultured astrocytes to proliferate...
and to produce proinflammatory cytokines (9, 27). Thus, it is conceivable that TWEAK-producing cells such as macrophages/microglia and astrocytes and Fn14-expressing astrocytes can form an amplification loop that perpetuates local inflammation and in which TWEAK can further trigger tissue damage through its various direct cytotoxic effects. In addition to stimulating the production of tissue-degrading enzymes such as metalloproteases (28), TWEAK can induce cell death of several cell types, including neurons under certain conditions (25), particularly in combination with cytokines such as IFN-γ (7, 8). Although the TWEAK and Fn14 expression patterns we observed highlight the 2 mechanisms previously mentioned, TWEAK possesses additional potentially disease-driving activities relevant to MS, including increasing brain-blood barrier permeability (28) and exacerbating demyelination (47). Accordingly, large numbers of TWEAK+ microglia were found in cortical lesions characterized by a profound and generalized type of tissue injury involving myelin, neurons, astrocytes, and blood vessels. The relationship between the degree of meningeal inflammation, high density of TWEAK-expressing cells, and extensive cell loss/damage in the more external layers of the cerebral cortex supports the view that cytotoxic factors that may diffuse from the inflamed meninges could synergize with TWEAK and, possibly, other cytotoxic factors produced in the brain parenchyma to induce the dramatic cortical pathology.

It is of interest that CD163, a scavenger receptor cytosteme-rich domain family member restricted to the monocyte/macrophage lineage, was recently shown to bind TWEAK (48). CD163 might act as a TWEAK scavenger in pathological conditions or serve as an alternate receptor for TWEAK in cells that lack Fn14 (48). In MS lesions, CD163 is mainly expressed in perivascular macrophages and intraparenchymal foamy macrophages and microglia (49), cells that we found to be largely negative for Fn14; this suggests a possible autocrine mechanism of regulation of TWEAK activity.

In conclusion, our current study represents the first detailed characterization of TWEAK/Fn14 expression patterns in a human disease context. The findings presented herein support a pathogenic involvement of TWEAK in MS that is mediated through Fn14 receptor expressed mostly on neurons and astrocytes and may contribute to multiple pathogenetic mechanisms. These conclusions are in agreement with the general notion that persistent TWEAK/Fn14 activation may have a detrimental role in diseases that are associated with chronic inflammation (19) and raise the possibility that TWEAK might be a target for therapeutic intervention and a useful biomarker for tissue damage in MS and other inflammatory/autoimmune diseases.

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