Altered Expression of CX3CL1 in Patients with Epilepsy and in a Rat Model

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Chemokine C-X3-C motif ligand 1 (CX3CL1, alias fractalkine), is highly expressed in the central nervous system and participates in inflammatory responses. Recent studies indicated that inflammatory processes within the brain constitute a common and crucial mechanism in the pathophysiological characteristics of epilepsy. This study investigated the expression pattern of CX3CL1 in epilepsy and its relationship with neuronal loss. Double immunolabeling, IHC, and immunoblotting results showed that CX3CL1 expression was upregulated in the temporal neocortex of patients with temporal lobe epilepsy. In a rat model of epilepsy, CX3CL1 up-regulation began 6 hours after epilepsy, with relatively high expression for 60 days. In addition, ELISA revealed that the concentrations of CX3CL1 in cerebrospinal fluid and serum were higher in epileptic patients than in patients with neurosis but lower than in patients with inflammatory neurological diseases. Moreover, H&E staining demonstrated significant neuronal loss in the brains of epileptic patients and in the rat model. Finally, the expression of tumor necrosis factor-related apoptosis-inducing ligand may play a role in CX3CL1-induced cell death. Thus, our results indicate that CX3CL1 may serve as a possible biomarker of brain inflammation in epileptic patients. (Am J Pathol 2012, 180:1950–1962; DOI: 10.1016/j.ajpath.2012.01.024)

Epilepsy is characterized by an enduring predisposition to seizures and by emotional and cognitive dysfunctions.1 This disorder affects approximately 50 million people worldwide and, therefore, is one of the most common neurological disorders.2 During the past 10 years, an increasing body of clinical and experimental evidence has provided strong support to the hypothesis that inflammatory processes within the brain might contribute to the pathophysiological characteristics of epilepsy.2 For instance, an increased expression of inflammatory markers (high-mobility group box-1,3 IL-1β,4 monocyte chemoattractant protein-1,5 NF-κB,6 and transforming growth factor-β type 1 receptor7) has been observed in surgically resected temporal tissues from patients with temporal lobe epilepsy (TLE).8 A similar inflammatory reaction has also been reported in experimental models of TLE. In particular, an increase of pro-inflammatory cytokines (IL-1β, IL-6, and tumor necrosis factor-α) has been detected in the rat hippocampus, starting within the first hour after the induction of a status epilepticus (SE) and lasting for several days.9

Chemokines are cytokines that orchestrate the traffic of leukocytes throughout the body.10 CX3CL1 (chemokine C-X3-C motif ligand/fractalkine, which is expressed in the central nervous system, participates in inflammatory responses in many brain disorders.11–13 CX3CL1 is the fourth chemokine type (CX3C motif), with three amino acid residues between the first and second cysteine.14 Unlike other chemokines, CX3CL1 is produced as a membrane-bound form presented at the cell surface by a mucinlike stalk. CX3CL1 can be released as a soluble form after proteolytic cleavage and is primarily localized in neurons and endothelial cells.15–19 Because of its characteristic structure, multiple properties, and cellular expression pattern, it has been hypothesized that CX3CL1 is involved in the intercellular communication among neurons, microglia, and endothelial cells, as well as in extravasation of leukocytes after brain injury and inflammatory central nervous system diseases.11,19,20

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CX3CL1 is likely to contribute to the inflammatory processes in epilepsy, to our knowledge, no study has addressed the expression of CX3CL1 in brain tissue of epileptic patients and rat models.

In this study, we investigated the expression of CX3CL1 in the temporal neocortex of patients with TLE, as well as the concentrations of CX3CL1 in cerebrospinal fluid (CSF) and serum from epileptic patients. To extend the results gained through the analysis of human tissues, the expression of CX3CL1 was investigated in the hippocampus and adjacent cortex of a rat model of TLE at different time points after epilepsy. The neuronal loss and expression of tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) after epilepsy were also examined.

**Materials and Methods**

**Human Brain Tissue and Clinical Data**

Thirty patients undergoing surgery for medically intractable TLE and 15 cases of nonepilepsy control were included in this study. All brain tissue was chosen at random from our epilepsy brain tissue bank. The brain tissue bank was reported in our previous studies.21–23 All procedures were performed with the formal consent of the patients or legal next of kin and were approved by the ethics committees of the respective institutions, according to the Declaration of Helsinki. Our study protocol complied with the guidelines for the conduct of research involving human subjects, as established by the NIH and the Committee on Human Research at Chongqing Medical University, Chongqing, China.

Presurgical assessment comprised a detailed history and neurological examination, interictal and ictal electroencephalographic studies, neuropsychological testing, and neuroradiological studies. At surgery, all patients with TLE were refractory to maximal doses of three or more anti-epileptic drugs, including carbamazepine, clonazepam, lamotrigine, phenobarbital, phenytoin, topiramate, and valproic acid. All tissue blocks were from the inpatient department, where computed tomography, magnetic resonance imaging, or CSF were further performed. The control group comprised 27 control subjects with inflammatory neurological diseases (bacterial meningitis, Bell’s palsy, Guillain-Barre syndrome, and viral meningitis) and 8 control subjects with neurosis.11,26–28 Neurosis is also termed neurotic disorders, which include somatization and anxiety disorders. Patients who presented with pain (eg, headaches, pain in the extremities, and back pain) or pseudoneurological symptoms (eg, difficulty swallowing, pseudoseizures, double or blurred vision, and blindness) were recruited in the inpatient department, where computed tomography, magnetic resonance imaging, or CSF were further performed, as indicated, to exclude possible underlying organic abnormalities. These patients were finally diagnosed as having neurosis or neuropathological examination revealed no signs of central nervous system disease. The mean ± SD age of the control group was 23.17 ± 11.52 years (range, 15 to 50 years). These subjects had no history of epilepsy or exposure to anti-epileptic drugs. There were no significant differences in age, sex, or topography of the studied tissues between TLE and control tissues.

**CSF and Serum**

**Patient Material**

All CSF and blood samples were obtained from the Department of Neurology (The First Affiliated Hospital, Chongqing Medical University).24,25 The CSF samples from 39 living epileptic patients were collected by lumbar puncture during conscious sedation. Patients were completely seizure free for >24 hours before CSF collection. The control group comprised 27 control subjects with inflammatory neurological diseases (bacterial meningitis, Bell’s palsy, Guillain-Barre syndrome, and viral meningitis) and 8 control subjects with neurosis. Bacterial meningitis, Bell’s palsy, Guillain-Barre syndrome, and viral meningitis were all considered inflammatory neurological diseases.11,26–28 Neurosis is also termed neuropathological examination revealed no signs of central nervous system disease. The mean ± SD age of the control group was 23.17 ± 11.52 years (range, 15 to 50 years). These subjects had no history of epilepsy or exposure to anti-epileptic drugs. There were no significant differences in age, sex, or topography of the studied tissues between TLE and control tissues.

**Table 1. Comparison of Clinical Data in the Patients with TLE and the Nonepileptic Controls**

<table>
<thead>
<tr>
<th>Clinical variable</th>
<th>TLE group (n = 30)</th>
<th>Control group (n = 15)</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>25.1 ± 12.3</td>
<td>23.2 ± 11.5</td>
<td>0.603</td>
</tr>
<tr>
<td>Range</td>
<td>8–58</td>
<td>15–50</td>
<td></td>
</tr>
<tr>
<td>Epilepsy onset (years)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>13.8 ± 9.8</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Range</td>
<td>1–38</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Epilepsy duration† (years)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>11.0 ± 7.7</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Range</td>
<td>1–29</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Seizure frequency (no./month)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>10.1 ± 6.9</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Range</td>
<td>3–30</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Seizure type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CPS</td>
<td>8</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>SGS</td>
<td>17</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>CPS and SGS</td>
<td>5</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Male/female ratio</td>
<td>16:14</td>
<td>9:6</td>
<td>0.757</td>
</tr>
</tbody>
</table>

*P values were computed using an independent-sample t-test (age) or a χ² test (male/female ratio). P < 0.05 was considered significant.
†Epilepsy duration was calculated as the time between epilepsy onset (onset of habitual seizures) and surgery. CPS, complex partial seizures; NA, not applicable; SGS, secondary generalized seizure.
Clinical characteristics are summarized in Table 5. Samples were both collected from the same patients. The other words, peripheral venous blood and lumbar CSF with neurosis) provided both CSF and serum samples. In epilepsy, 11 with inflammatory neurological diseases, and 8

tubes were cooled immediately after sampling, centrifuged, weren't written informed consent was obtained from all partic-

ticipants.

The study was approved by the Committee on Hu-

man Research at Chongqing Medical University, and written informed consent was obtained from all partic-

pants.

CSF and Serum Studies

The CSF and venous blood samples drawn in EDTA tubes were cooled immediately after sampling, centrifuged, and stored frozen at −80° until analysis, as previously described.24 CX3CL1 was measured with commercially avail-

able ELISA kits (Human CX3CL1/Fractalkine Immunoassay, number DCX310; R&D Systems, Minneapolis, MN). Values were calculated from a standard curve generated for each ELISA result. Samples were not diluted, and results were standardized according to previously established protein concentrations, with the final concentration expressed as ng/mL protein.

Rat Model of Epilepsy

All animal procedures were approved by the Commission of Chongqing Medical University for ethics of experi-
ments on animals and were conducted in accordance with international standards.

The rat model was made as previously reported in our laboratory.22,23 Healthy adult male Sprague-Dawley rats (n = 49) from Chongqing Medical University Laboratory Animal Center, weighing 200 to 250 g, were randomly divided into the normal control group (n = 7) or the experimental group (n = 42). The experimental group was randomly divided into six subgroups: 6 hours, 72 hours, 7 days, 14 days, 30 days, and 60 days after SE. Rats were injected with lithium chloride (127 mg/kg, i.p.; Sigma-Aldrich, St. Louis, MO). Approximately 18 hours later, atropine sulfate (1 mg/kg, i.p.) was administered to limit the peripheral effects of the convulsant. Thirty minutes later, SE was induced by injecting pilocarpine hydrochloride (30 mg/kg, i.p.; Sigma) in 42 rats. Pilocarpine hydrochloride was given repeatedly (10 mg/kg, i.p.) every 30 minutes until the rats developed seizures. At 6 to 11 days after pilocarpine, these rats would develop spontaneous recurrent seizures. The evoked seizures were scored according to Racine.29 Only those rats that attained stage 4 to 5 were taken into the study. Their seizure frequency was five to eight per week. All rats were video monitored continuously starting immediately after pilocarpine injection until the day they were sacrificed. Seven control rats received the same treat-

ment with lithium chloride and atropine sulfate, but we used saline instead of pilocarpine. At 1 hour after SE onset, we reduced the severity of convulsions with 10 mg/kg diazepam, i.p. The experimental animals were sacrificed 6 hours, 72 hours, 7 days, 14 days, 30 days, or 60 days after SE, and the hippocampus and adjacent cortex were removed for study.

Table 2. Clinical Features of the Epileptic Patients in the CSF Study

<table>
<thead>
<tr>
<th>Clinical variable</th>
<th>Epilepsy group (n = 39)</th>
<th>Inflammation group (n = 27)</th>
<th>Neurosis group (n = 8)</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>29.4 ± 11.3</td>
<td>34.8 ± 12.6</td>
<td>33.4 ± 12.3</td>
<td>0.190</td>
</tr>
<tr>
<td>Range</td>
<td>15–60</td>
<td>16–60</td>
<td>18–55</td>
<td></td>
</tr>
<tr>
<td>Male/female ratio</td>
<td>22:17</td>
<td>13.14</td>
<td>4.4</td>
<td>0.793</td>
</tr>
</tbody>
</table>

*P values were computed using a one-way analysis of variance (age) or a χ² exact test (male/female ratio). P < 0.05 was considered significant.

Table 3. Clinical Features of the Epileptic Patients in the CSF and Serum Studies

<table>
<thead>
<tr>
<th>Clinical variable</th>
<th>CSF group (n = 39)</th>
<th>Serum group (n = 43)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>29.4 ± 11.3</td>
<td>30.0 ± 11.7</td>
</tr>
<tr>
<td>Range</td>
<td>15–60</td>
<td>16–60</td>
</tr>
<tr>
<td>Seizure frequency (no./month)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>6.6 ± 5.2</td>
<td>6.9 ± 4.8</td>
</tr>
<tr>
<td>Range</td>
<td>0.05–20</td>
<td>0.05–20</td>
</tr>
<tr>
<td>Seizure duration (years)*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 5</td>
<td>21</td>
<td>19</td>
</tr>
<tr>
<td>5–10</td>
<td>11</td>
<td>16</td>
</tr>
<tr>
<td>10–20</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>Seizure type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CPS</td>
<td>15</td>
<td>17</td>
</tr>
<tr>
<td>GTCS</td>
<td>7</td>
<td>9</td>
</tr>
<tr>
<td>SGS</td>
<td>17</td>
<td>17</td>
</tr>
<tr>
<td>Male/female ratio</td>
<td>22:17</td>
<td>23:20</td>
</tr>
</tbody>
</table>

Data are given as number of patients unless otherwise indicated.

*Epilepsy duration was calculated as the time between epilepsy onset (onset of habitual seizures) and sample collection.

CPS, complex partial seizure; GTCS, generalized tonic-clonic seizure; SGS, secondary generalized seizure.
**Table 4. Clinical Features of the 78 Patients in the Serum Study**

<table>
<thead>
<tr>
<th>Clinical variable</th>
<th>Epilepsy group (n = 43)</th>
<th>Inflammation group (n = 26)</th>
<th>Neurosis group (n = 9)</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>30.0 ± 11.7</td>
<td>31.3 ± 11.5</td>
<td>31.7 ± 12.6</td>
<td>0.873</td>
</tr>
<tr>
<td>Range</td>
<td>16–60</td>
<td>16–60</td>
<td>18–55</td>
<td></td>
</tr>
<tr>
<td>Male/female ratio</td>
<td>23:20</td>
<td>12:14</td>
<td>4:5</td>
<td>0.789</td>
</tr>
</tbody>
</table>

*P values were computed using a one-way analysis of variance (age) or a χ² exact test (male/female ratio). P < 0.05 was considered significant.

**Tissue Processing**

For both human and animal tissues, one portion of resected brain tissue was immediately fixed in 10% buffered formalin for 48 hours. Tissues were then embedded in paraffin, divided into sections for immunohistochemistry (IHC; 5 μm thick) and for double-immunofluorescence labeling analysis (10 μm thick). For neuropathological evaluation, representative paraffin sections (5 μm thick) (two sections of each specimen of human and five sections of each animal) were stained with H&E. Other portions of the resected brain tissues were immediately stored in liquid nitrogen and later used for protein extraction (see the protocol for Western blot analysis). Animals were perfused transcardially with physiological saline, followed by 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4) under chloral hydrate anesthesia (0.35 g/kg, i.p.). The brains were removed and postfixed in the same fixative for 1 hour. Thereafter, the entire hippocampus and adjacent cortex were frozen and divided into sections with a cryostat (10 μm thick) for double-immunofluorescence labeling analysis.

**Double-Immunofluorescence Labeling**

Tissue sections were deparaffinized, rehydrated in a graded series of ethanol, and then incubated in H₂O₂ (0.3%, 15 minutes). Frozen sections were air dried on a slide warmer. The slides were immersed in 0.06% potassium permanganate for 15 minutes and then incubated in 5% goat serum for 1 hour at room temperature. Sections were then incubated with a mixture of polyclonal rabbit anti-CX3CL1 antibody (1:100, catalogue number ab25088; Abcam, Cambridge, MA) or with a mixture of polyclonal rabbit anti-gliarial fibrillary acidic protein (GFAP) antibody (Wuhan Boster Biological Technology) at 4°C overnight. Sections were washed and incubated with fluorescein isothiocyanate–conjugated goat anti-rabbit IgG (1:200; Zhongshan Golden Bridge Inc., Beijing, China) and tetramethylrhodamine isothiocyanate–conjugated goat anti-mouse IgG (1:200; Zhongshan Golden Bridge Inc.) in the dark for 60 minutes at room temperature, and then mounted in 50% glycerol/PBS. For TRAIL immunofluorescence, sections were incubated with a mixture of polyclonal rabbit anti-TRAIL antibody (1:50, BA1446; Wuhan Boster Biological Technology) and mouse anti-MAP2 antibody (Wuhan Boster Biological Technology) or with a mixture of polyclonal rabbit anti-TRAIL antibody and mouse anti-GFAP antibody (Wuhan Boster Biological Technology) at 4°C overnight. The secondary antibodies were fluorescein isothiocyanate–conjugated goat anti-mouse IgG (1:50, Beijing CoWin Bioscience Co, Ltd, Beijing) and tetramethylrhodamine isothiocyanate–conjugated goat anti-rabbit IgG (1:50; CoWin Bioscience Beijing, China).

Fluoro-Jade B (FJB) staining was used to identify degenerating neurons in tissues obtained from animals. Briefly, sections were single immunofluorescence labeled of TRAIL (polyclonal rabbit anti-TRAIL antibody and tetramethylrhodamine isothiocyanate–conjugated goat anti-rabbit IgG were used) and then dried on a slide warmer. The slides were immersed in 0.06% potassium permanganate for 15 minutes and gently agitation. After rinsing in distilled water for 2 minutes, the slides were incubated for 20 minutes in 0.0004% polyanion fluorescent derivative solution (AG310, FJB; Millipore Corporation, Billerica, MA), freshly prepared by adding 4 mL of a 0.01% stock FJB solution to 96 mL of 0.1% acetic acid, with gentle shaking in the dark. After rinsing for 1 minute in each of three changes of distilled water, the slides were dried and coverslipped with 50% glycerol/PBS. Fluorescence was detected by laser-scanning confocal microscopy (Leica Microsystems Heidelberg GmbH, Wetzlar, Germany) on an Olympus IX70 inverted microscope (Olympus, Tokyo, Japan) equipped with a Fluoview FVX.

**Table 5. Clinical Features of the 40 Patients in the CSF/Serum Study**

<table>
<thead>
<tr>
<th>Clinical variable</th>
<th>Epilepsy group (n = 21)</th>
<th>Inflammation group (n = 11)</th>
<th>Neurosis group (n = 8)</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>26.9 ± 10.0</td>
<td>33.5 ± 14.5</td>
<td>33.4 ± 12.3</td>
<td>0.232</td>
</tr>
<tr>
<td>Range</td>
<td>16–54</td>
<td>16–60</td>
<td>18–55</td>
<td></td>
</tr>
<tr>
<td>Male/female ratio</td>
<td>12:9</td>
<td>4.7</td>
<td>4.4</td>
<td>0.536</td>
</tr>
</tbody>
</table>

*P values were computed using a one-way analysis of variance (age) or a χ² exact test (male/female ratio). P < 0.05 was considered significant.
confocal scan head (Leica Microsystems Heidelberg GmbH).

**IHC Analysis**

IHC staining was conducted using the avidin-biotin-peroxidase complex method, according to established protocols or recommendations by the manufacturers. The primary antibody was rabbit anti-CX3CL1 (catalogue number ab25088; Abcam), and dilution was 1:100. The secondary antibody (Wuhan Boster Biological Technology) kit was used. For negative controls, the primary antibodies were replaced with PBS. A slide image resulting from each section was scanned and acquired by an OLYMPUS PM20 automatic microscope (Olympus, Tokyo, Japan) and a TCFY-2050 (Yuancheng Inc., Beijing, China) pathology system. Ten visual fields in each sectional image were obtained randomly (five sections in each brain). By using the Motic Med 6.0 CMIAS pathology image analysis system (Beihang Motic Inc., Beijing, China), HIC results were assessed by automatically measuring the average integrated OD that was calculated as integrated OD over field area ratio. The mean value from 10 fields in each slide image was thus collected and used to measure the differences between epilepsy and control. For TRAIL IHC, the antibody of rabbit anti-TRAIL (1:50, BA1446; Wuhan Boster Biological Technology) was used.

**Western Blot Analysis**

Proteins, 50 µg, were separated by 10% SDS-PAGE and then transferred to a polyvinylidene fluoride membrane (Millipore Corporation) for Western blot analysis using a Bio-Rad apparatus (Bio-Rad Laboratories, Richmond, CA). Nonspecific epitopes were blocked with 5% skim milk/Tween-20–Tris-buffered saline. The membranes were incubated overnight at 4°C with each of the following primary antibodies: rabbit anti-CX3CL1 antibody (1:300, catalogue number ab25088; Abcam), goat anti-TRAIL antibody (1:100, sc-6079; Santa Cruz Biotechnology Inc., Santa Cruz, CA), and rabbit anti-β-actin as control antibody (1:5000; Beijing 4A Biotech Co, Ltd, Beijing). After three washes in Tween-20–Tris-buffered saline (TBST), the membranes were then treated with horseradish peroxidase–conjugated secondary antibodies for 1 hour at room temperature and revealed with an Enhanced Chemiluminescence Kit (Pierce, Rockford, IL) and a charge-coupled device camera (Bio-Rad Laboratories) in a dark room, digitally scanned immune blots were analyzed using Quantity One software version 4.6.2 (Bio-Rad Laboratories). Band immune intensity ratios of CX3CL1 or TRAIL and corresponding β-actin at the same time of electrophoresis were analyzed; the ratios were the average OD values of CX3CL1 or TRAIL blot expression.

**Cell Quantification for H&E Staining**

Ten visual fields of each section were randomly chosen under a light microscope. Cell quantification was performed as described elsewhere. Briefly, areas or numbers of labeled cells in each section were traced and measured using an image analysis system (Image-Pro Plus Media Cybernetics, Silver Spring, MD). Slides were first examined at ×100 magnification to identify

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**Figure 1.** Double-immunofluorescent labeling, IHC, and Western blot analysis for CX3CL1 in the temporal neocortex of patients with TLE and nonepileptic patients. A: Double-immunofluorescent labeling shows that the chemokine CX3CL1 (green) and MAP2 (red) are coexpressed (merged) in the temporal neocortex of an epileptic patient. White arrows, CX3CL1+/MAP2+ cells. B: IHC analysis for CX3CL1 in the temporal neocortex of humans demonstrating immunoreactive staining of CX3CL1 in the temporal neocortex of a control subject compared with strong immunoreactive staining of CX3CL1 in the temporal neocortex of a patient with TLE. Black arrows, CX3CL1+ cells. Comparison of the mean OD value (right) indicates significantly higher expression of CX3CL1 in the TLE group than in the control group. *P < 0.05. C: Western blotting analysis for CX3CL1 in the temporal neocortex of humans. Proteins from individual brain homogenates from TLE and control subjects were separated with gradient SDS-PAGE. CX3CL1 was more strongly expressed in patients with TLE than in controls. A comparison of the intensity ratio (right) indicates significantly higher expression of CX3CL1 in patients with TLE than in controls. *P < 0.05.
the regions and then labeled cell numbers were counted at ×400 magnification. Surviving neurons were counted in H&E-stained sections (5 μm thick). Cells with somas <3 μm were considered to be glial or necrotic cells and were excluded. The percentage of area occupied by H&E-stained neuronal nuclei was calculated for each image, and the mean value for each animal was determined by averaging values from all images taken from that animal. The mean value observed in control rats was designated as 100% of the normal cell population.

Statistical Analysis

Data were expressed as mean ± SD, and the analysis was performed using the Student’s t-test (SPSS 11.5; SPSS Inc., Chicago, IL) between the TLE and control groups. The statistical differences in CSF and serum analyses were determined by one-way analysis of variance, combined with a post hoc Bonferroni test as a multiple comparison method, as was the statistical difference among groups of experimental animals. P < 0.05 was considered statistically significant.

Results

Neuronal Localization of CX3CL1

By using double-immunofluorescence labeling, we found CX3CL1 expressed exclusively in neurons of temporal neocortex tissue from nonepileptic control and TLE patients, as shown by colocalization with the dendritic marker, MAP2 (Figure 1A). GFAP+ astrocytes were not stained (data not shown), indicating that CX3CL1 was not expressed in the astrocytes.

Elevated CX3CL1 Protein Expression in the Temporal Neocortex of Patients with TLE

CX3CL1 protein was expressed in the membrane and cytoplasm of neurons in the temporal neocortex of tissue from nonepileptic autopsy control and TLE patients, faint CX3CL1 staining was present in sections from the nonepileptic autopsy group, and strong immunoreactivity for CX3CL1 was observed in TLE samples (Figure 1B). No immunoreactivity was seen in negative controls in which the primary antibody had
been omitted (data not shown). The mean OD value of CX3CL1 protein in the temporal neocortex tissue of patients with TLE was significantly higher than that of the nonepileptic autopsy group (0.73 ± 0.25 versus 0.30 ± 0.13; \( P < 0.01 \)) (Figure 1B).

The Western blot analysis result was in accordance with that of IHC: CX3CL1-immunoreactive bands were seen at approximately 95 kDa, and β-actin–immunoreactive bands were seen at 42 kDa. We evaluated the expression of CX3CL1 in the temporal neocortex of all TLE and nonepileptic controls. These results confirmed the slight basal level of CX3CL1 in the temporal neocortex of nonepileptic patients and its overexpression in patients with TLE (Figure 1C). The difference in the mean OD value between the TLE group and the nonepileptic control group was statistically significant (1.32 ± 0.21 versus 0.68 ± 0.11; \( P < 0.01 \)) (Figure 1C).

Elevated CX3CL1 Concentrations in Serum and CSF in Epilepsy

We first studied the CSF concentrations of CX3CL1 in 39 patients with epilepsy, 27 control subjects with inflammatory neurological diseases, and 8 control subjects with neurosis. The level of CX3CL1 was significantly elevated in CSF samples from patients with epilepsy compared with patients with neurosis (\( P < 0.05 \)) (Figure 2, A and B). In addition, the CX3CL1 level was increased in CSF samples from patients with inflammatory neurological diseases compared with both other groups (\( P < 0.05 \)) (Figure 2, A and B) (epilepsy group, 0.23 ± 0.12 ng/mL; inflammatory neurological diseases group, 0.32 ± 0.15 ng/mL; neurosis group, 0.09 ± 0.07 ng/mL) (Figure 2B).

Then, we studied the serum concentrations of CX3CL1 in 43 patients with epilepsy, 26 control subjects with inflammatory neurological diseases, and 9 control subjects with neurosis. The serum concentrations of CX3CL1 differed in patients with epilepsy, inflammatory neurological diseases, and neurosis (\( P < 0.01 \)) (Figure 2, C and D). Patients with epilepsy had a higher serum concentration of CX3CL1 than patients with neurosis (0.85 ± 0.32 versus 0.41 ± 0.16 ng/mL; \( P < 0.01 \)) (Figure 2D). Patients with inflammatory neurological diseases had a higher concentration of CX3CL1 than patients with epilepsy (0.85 ± 0.32 versus 0.12 ± 0.48 ng/mL; \( P < 0.01 \)) (Figure 2D). The differences in CSF-CX3CL1 and serum-CX3CL1 levels in different age or sex subgroups in patients with epilepsy were not statistically significant (data not shown) (\( P > 0.05 \)). Moreover, there were no significant differences in CSF or serum concentrations of CX3CL1 in epileptic patients with different disease durations (data not shown) (\( P > 0.05 \)).

The 40 CSF samples and 40 serum samples were collected from the same set of 40 patients. We analyzed the ratios of CSF concentrations of CX3CL1 to serum concentrations of CX3CL1 in the same patients. There were no significance differences in the ratios in patients with epilepsy, patients with inflammatory neurological diseases, and patients with neurosis (epilepsy group, 0.28 ± 0.14; inflammatory neurological diseases group, 0.35 ± 0.16; neurosis group, 0.25 ± 0.18; \( P > 0.05 \)) (Figure 2, E and F). These results indicate that elevated serum CX3CL1 was presented in epilepsy. Blood-brain

![Figure 3](image-url)

**Figure 3.** The CSF and serum CX3CL1 levels in epileptic patients with different seizure frequency subgroups. A: The CSF levels of CX3CL1 in epileptic patients with different seizure frequency subgroups. The concentrations of CX3CL1 in different seizure frequency subgroups are not significantly different: <5, 0.19 ± 0.11 ng/mL; 5 to 10, 0.25 ± 0.13 ng/mL; and 10 to 20, 0.31 ± 0.12 ng/mL. \( P > 0.05 \). B: Serum levels of CX3CL1 in epileptic patients with different seizure frequency subgroups are not significantly different: <5, 0.76 ± 0.31 ng/mL; 5 to 10, 0.88 ± 0.24 ng/mL; and 10 to 20, 1.02 ± 0.43 ng/mL. \( P > 0.05 \).
Barrier (BBB) leakage is associated with seizure attacks, which may contribute to blood-brain exchanges of CX3CL1. To further clarify if the increased serum CX3CL1 comes from dysfunctional brain tissues through disrupted BBB, we assessed CSF to blood albumin ratios in these three groups. Similar to those of CX3CL1, CSF to blood ratios of albumin were not significantly changed (0.0026 ± 0.0010 in epilepsy, n = 7; 0.0056 ± 0.0031 in inflammation, n = 10; and 0.0035 ± 0.0027 in neurosis, n = 4; P > 0.05; N = 21).

To assess if the seizure frequency may have affected CX3CL1 level, we measured CSF/blood concentrations according to seizure frequencies. The seizure frequency of epileptic patients was between 0.5 and 20 times per month. We divided the epileptic patients into three subgroups: <5, 5 to 10, and 11 to 20 times per month. There was no difference of CX3CL1 in different seizure frequency subgroups between CSF and serum (P > 0.05; Figure 3, A and B).

Prominent CX3CL1 Expression in the Hippocampus and Adjacent Cortex in the Epileptic Rats

To exclude the possibility that altered CX3CL1 expression may be caused by anti-epileptic drugs in patients with epilepsy, we performed an experiment in a rat model of epilepsy. CX3CL1 was expressed exclusively in neurons of the hippocampus and adjacent cortex from control and epileptic rats, as shown by colocalization with the dendritic marker, MAP2 (Figure 4A). GFAP+ astrocytes were not stained (Figure 4B). CX3CL1 was not expressed in the astrocytes.

CX3CL1 was expressed in the membrane and cytoplasm of neurons of the hippocampus and adjacent cortex (Figure 4, A–C). In the epileptic groups, we observed stronger staining for CX3CL1 in neurons of the granule cell layer of the dentate gyrus, CA1 and CA3 pyramidal cell layers, and adjacent cortex (Figure 4C). IHC analysis showed that the mean OD value of each time point after treatment.
SE was significantly higher than that of the control group ($P < 0.01$). We found an increase in CX3CL1 expression at 72 hours (298.3% of that in controls, $P < 0.01$), at 14 days (248.5% of that in controls, $P < 0.01$), and at 60 days (195.0% of that in controls, $P < 0.01$) (Figure 4C).

Western blot analysis was performed to further verify the elevated CX3CL1 immunostaining observed in epileptic rat brain sections. In accordance with IHC, CX3CL1 up-regulation in epileptic tissue appeared at 6 hours after SE, reached a high level at 72 hours, and was maintained at a relatively high level until 60 days (Figure 4D). Semiquantitative densitometric analysis revealed that, in brain tissue, CX3CL1 expression of each time point after SE was significantly increased compared with the control group ($P < 0.01$) (Figure 4D). We found an increase in CX3CL1 expression at 72 hours (186.6% of that in controls, $P < 0.01$) and at 14 days (165.1% of that in controls, $P < 0.01$) after SE (Figure 4D).

Neuronal Loss in the Epileptic Patients and Rats

The H&E staining demonstrated that the anatomical sites of the samples were located in the temporal neocortex. The number of neurons was reduced in the temporal neocortex of patients with TLE compared with the number of neurons in the control tissues ($P < 0.05$). Neuron loss and gliosis were seen in the temporal neocortex of patients with TLE (Figure 5, A–C). The number of neurons in the temporal neocortex of patients with TLE was 53.67% of that in controls (Figure 5D).

In a rat model of epilepsy, we counted the number of surviving neurons in the hippocampus and adjacent cortex of control rats and rats after SE at representative time points: 72 hours, 7 days, and 60 days. H&E staining demonstrated that the number of neurons was reduced in the hippocampus and adjacent cortex of the epileptic rats compared with the number of neurons in control rats. Neuron loss and gliosis were seen in the hippocampus and adjacent cortex of the epileptic rats. We found a decrease in the number of surviving neurons at 72 hours (89.34% of that in controls, $P < 0.01$), 7 days (83.26% of that in controls, $P < 0.01$), and 60 days (61.75% of that in controls, $P < 0.01$) after SE (Figure 5).

Prominent TRAIL Expression in Epileptic Patients and Rats

TRAIL was expressed in the membrane and cytoplasm of cells of the temporal neocortex of patients (Figure 6A).
Western blot analysis study showed that TRAIL was significantly increased in epileptic patients (Figure 6B). TRAIL-immunoreactive bands were seen at approximately 34 kDa, and β-actin–immunoreactive bands were seen at 42 kDa. We evaluated the expression of TRAIL in the temporal neocortex of patients with TLE and nonepileptic controls. Temporal neocortex samples from patients with TLE showed stronger TRAIL immunoreactivity when compared with normal brain tissue from nonepileptic autopsy controls (Figure 6B). The difference in mean OD values between the TLE and nonepileptic control groups was statistically significant (1.23 ± 0.52 versus 0.47 ± 0.15; P < 0.01) (Figure 6B).

TRAIL was expressed in the membrane and cytoplasm of neurons and astrocytes in the hippocampus and adjacent cortex of rats (Figure 7, A and B). We found TRAIL expressed in neurons and astrocytes in epileptic rats, as shown by colocalization with the dendritic marker, MAP2 (Figure 7A), or the astrocyte marker, GFAP (Figure 7A), indicating that TRAIL was expressed in both neurons and astrocytes. Colocalization of TRAIL with FJB-stained cells suggests that TRAIL is included in injured neurons (Figure 7A).

Western blot analysis was performed to evaluate the expression of TRAIL in the hippocampus and adjacent cortex of epileptic rats. TRAIL up-regulation in epileptic tissue appeared at 6 hours after SE, gradually increased along with the time after seizures, and reached a high level at 60 days (Figure 7B). Semiquantitative densitometric analysis revealed that, in brain tissue, TRAIL expression of each time point after SE was significantly increased compared with the control group (P < 0.05) (Figure 7C).

Discussion

The major findings of this study are as follows: i) CX3CL1 is significantly up-regulated in neurons from patients with TLE and lithium chloride–pilocarpine–induced rats; ii) CSF and serum concentrations of CX3CL1 are significantly increased in patients with epilepsy; iii) the neuronal loss is apparent in epileptic patients and rats; and iv) consistent with neuronal death, TRAIL is significantly increased in both patients and the animal model.

Although the underlying mechanisms of epileptogenesis are still unknown, inflammation mechanisms, such as pro-inflammatory cytokines, play a critical role in the pathogenesis of epilepsy. Chemokines can modulate neuronal activity, as follows: i) modulation of voltage-dependent channels (sodium, potassium, and calcium); ii) activation of the G-protein–activated inward rectifier potassium current; and iii) increase of neurotransmitter release (GABA, glutamate, and dopamine), often through calcium-dependent mechanisms. In the present study, CX3CL1 expression was up-regulated in patients and an animal model. Epilepsy involves molecular factors, cellular alterations, and neuronal network reorganization. Increased expression of CX3CL1 may control neuronal excitability by modulating glutamatergic α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) currents, although its exact role in the epilepsy phenotype remains unclear.

Consistent with the finding in the brain tissue, our research showed that levels of CX3CL1 in CSF and serum of patients with epilepsy were significantly higher than those in patients with neurosis. However, we did not find an increased CSF/serum ratio in patients versus controls. The increased serum CX3CL1 may be attributed to BBB leakage in epilepsy. BBB disruption and angiogenesis are associated with TLE and an experimental animal model. Evidence has also shown that BBB leakage increases seizure frequency. An impaired tight junction may allow circulating IgG to be accumulated in neurons through a compromised BBB, which may contribute to epileptogenesis. Conversely, increased expression of brain fractalkine gains access to the blood flow and results in an elevated serum level, although direct evidence is lacking. Therefore, we assume that augmentation of
fractalkine in both CSF and serum resulted in relatively unchanged CSF/serum ratios. The less CX3CL1 in epilepsy than in inflammatory central nervous system diseases suggests that inflammatory processes in epilepsy may not be a prominent feature (Figure 2).\(^\text{11}\)

In this study, the number of neurons was reduced after epilepsy, which is consistent with previous studies.\(^\text{21,38,39}\) Evidence indicates that cell death and inflammation parallel the time course of hippocampal remodeling after seizure. In support of this, intracerebroventricular infusions of recombinant rat CX3CL1 aggravated SE-induced neuronal damage.\(^\text{40}\) Both necrosis and apoptosis may contribute to neuronal loss, depending on seizure activity and cell vulnerability. Although necrosis is prominent in SE, a condition in which repetitive seizure activity lasts for tens to hundreds of minutes,\(^\text{41}\) apoptosis is associated with both SE and multiple brief seizures.\(^\text{42,43}\) On the other hand, different neuron populations may have varied vulnerability. Prolonged afferent stimulation leads to necrosis in hilar neurons and pyramidal cells in the hippocampus, but the same stimulation causes apoptosis-like morphological features in dentate granule cells.\(^\text{44}\)

Up-regulation of inflammation mediators IL-1, tumor necrosis factor, and IL-6 after seizures contribute to neuronal death.\(^\text{2,45}\) However, whether TRAIL is also deregulated in epilepsy is relatively unclear. Our study showed that TRAIL was significantly increased in patients and an animal model. Colocalization of TRAIL with FJB-stained cells suggests that TRAIL is involved in cell death in epilepsy. Surprisingly, fractalkine has been protective. Incubation of fractalkine in hippocampal slices depresses excitatory postsynaptic current and AMPA-type glutamate receptor current, whereas fractalkine in both CSF and serum resulted in relatively unchanged CSF/serum ratios. The less CX3CL1 in epilepsy than in inflammatory central nervous system diseases suggests that inflammatory processes in epilepsy may not be a prominent feature (Figure 2).\(^\text{11}\)

Up-regulation of inflammation mediators IL-1, tumor necrosis factor, and IL-6 after seizures contribute to neuronal death.\(^\text{2,45}\) However, whether TRAIL is also deregulated in epilepsy is relatively unclear. Our study showed that TRAIL was significantly increased in patients and an animal model. Colocalization of TRAIL with FJB-stained cells suggests that TRAIL is included in injured neurons (Figure 7A). There was a trend that expression of TRAIL was increased along with the time after seizures, which was consistent with that of fractalkine. These results suggest that TRAIL is involved in cell death in epilepsy. Surprisingly, fractalkine has been protective. Incubation of fractalkine in hippocampal neurons promotes survival.\(^\text{17}\) Bath application of fractalkine in hippocampal slices depresses excitatory postsynaptic current and AMPA-type glutamate receptor current, whereas fractalkine in both CSF and serum resulted in relatively unchanged CSF/serum ratios. The less CX3CL1 in epilepsy than in inflammatory central nervous system diseases suggests that inflammatory processes in epilepsy may not be a prominent feature (Figure 2).\(^\text{11}\)
Altered Expression of CX3CL1 in Epilepsy

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Acknowledgments

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