Dynamic Change in Cells Expressing IL-1β in Rat Hippocampus after Status Epilepticus

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ABSTRACT

BACKGROUND: The time course of cytokine dynamics after seizure remains controversial. Here we evaluated the changes in the levels and sites of interleukin (IL)-1β expression over time in the hippocampus after seizure.

METHODS: Status epilepticus (SE) was induced in adult Wistar rats by means of intraperitoneal injection of kainic acid (KA). Subsequently, the time courses of cellular localization and IL-1β concentration in the hippocampus were evaluated by means of immunohistochemical and quantitative assays.

RESULTS: On day 1 after SE, CA3 pyramidal cells showed degeneration and increased IL-1β expression. In the chronic phase (>7 days after SE), glial fibrillary acidic protein (GFAP)—positive reactive astrocytes—appeared in CA1 and became IL-1β immunoreactive. Their IL-1β immunoreactivity increased in proportion to the progressive hypertrophy of astrocytes that led to gliosis. Quantitative analysis showed that hippocampal IL-1β concentration progressively increased during the acute and chronic phases.

CONCLUSION: IL-1β affects the hippocampus after SE. In the acute phase, the main cells expressing IL-1β were CA3 pyramidal cells. In the chronic phase, the main cells expressing IL-1β were reactive astrocytes in CA1.

KEYWORDS: interleukin-1β, status epilepticus, hippocampus, gliosis, reactive astrocyte

Introduction

Hippocampal gliosis, which is caused by pyramidal cell death induced by prolonged febrile convulsions, is an epileptic focus in patients with temporal lobe epilepsy (TLE).1,2 In experimental studies, rats with kainic acid (KA)-induced status epilepticus (SE) develop spontaneous and recurrent seizures as well as pathologic hippocampal changes similar to those associated with human TLE; they are therefore considered an appropriate animal model of TLE.3-6 Human and animal studies have both shown that inflammatory molecules, including cytokines, are key contributors to the seizure-associated progression of hippocampal gliosis.7-10 Interleukin (IL)-1β is a major pro-inflammatory cytokine synthesized by macrophages, glial cells, and neuronal cells during infection and inflammatory processes.11-13 Rats with KA-induced SE receiving intranasal administration of IL-1β showed repeated and prolonged hyperthermia-induced seizures with a significantly reduced onset time.14 Furthermore, intrahippocampal injection of IL-1β extends the duration of KA-induced SE.15,16 In contrast, treatment with an exogenous IL-1 receptor antagonist or overexpression of its endogenous form markedly inhibits neuronal injury induced by bicuculline or cerebral ischemia.17,18
Pretreatment with an IL-1β receptor antagonist significantly reduces the onset of pilocarpine-induced SE and damage to the blood–brain barrier in rats.19

A previous immunohistochemical study has shown that in the acute phase after KA administration, IL-1β is expressed in the stratum oriens and stratum radiatum of both the CA1 and CA3 areas of the hippocampus.16 Another study has found diffuse IL-1β immunoreactivity throughout the hippocampus one to two days after SE.9,16 By contrast, IL-1β expression is present on microglial cells in the granule and molecular cell layers as well as the hilum of the dentate gyrus within 24 hours after SE.9,16 In the chronic phase (>7 days) after SE, reactive astrocytes in CA3 begin to express IL-1β,10,20 as do some microglia in the same region at four weeks. Another report has demonstrated that glia in CA3 expressed IL-1β at 7 days, but not at 60 days, after SE in two of six spontaneously epileptic rats.9

Although this evidence supports an important role of IL-1β in the pathogenesis of both human and animal TLE, the cells expressing IL-1β in the development of hippocampal sclerosis and neuronal excitability vary depending on the study, and therefore their identity remains unclear. To determine which cells are affected by IL-1β in the process of hippocampal glial formation after SE, we investigated IL-1β expression in the hippocampus, focusing on pyramidal and glial cells at various time points after SE.

Materials and Methods

Animals and treatments. The animal committee of Osaka City University School of Medicine approved this study, which was conducted in accordance with the Guidelines for Use and Care of Experimental Animals. Eight-week-old male Wistar rats (180–200 g) were provided food and water ad libitum under a standard 12:12-hour light-dark cycle. Rats were injected first with KA (10 mg/kg) intraperitoneally to induce SE, and then two hours after seizure onset with diazepam (10 mg/kg) to abort the SE.5 Seizure severity was determined by using the Racine scale: stage 1, mouth and facial movements; stage 2, head nodding; stage 3, forelimb clonus; stage 4, rearing; and stage 5, rearing and falling.21 Four control rats received saline. A total of 16 rats were anesthetized with sodium pentobarbital on days 1 (12–24 hours after SE), 7, 14, and 21 (n = 4 at each time point) after KA administration and then transcardially perfused with phosphate-buffered saline (PBS, pH 7.4). A total of three rats died owing to SE (one each on days 1, 7, and 14).

After sacrifice, the brains of the rats were quickly removed. The right hemisphere of each brain was post-fixed by immersion in 4% paraformaldehyde overnight at 4°C, then embedded in Tissue-Tek optimum cutting temperature (OCT) compound (Miles, Elkhart, IN, USA), frozen with liquid nitrogen, and sliced into coronal frozen sections (thickness, 20 μm) with a cryostat. The left hemisphere of each brain was immediately prepared for quantitative analysis of IL-1β as detailed below.

IL-1β immunohistochemistry. Several sections from each rat brain were examined for IL-1β localization by means of an immunohistochemical approach; other sections were assessed for the co-localization of IL-1β and glial fibrillary acidic protein (GFAP) by means of a double-label immunofluorescence technique. For the immunohistochemical study, a commercially available rabbit polyclonal anti-IL-1β antibody (diluted 1:500; sc-1251, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used. An anti-human monoclonal GFAP antibody (diluted 1:50; 03–61011, American Research Products, Belmont, MA, USA) was used in the assessment of GFAP localization. Double-label immunofluorescence staining of IL-1β (sc-1251 diluted 1:250) and GFAP (03–61011), as well as immunohistochemistry, was carried out as described previously.5 To assess the specificity of the primary antibodies, additional sections were subjected to immunohistochemical processing without primary antibody and used as negative controls; these sections were also stained with hematoxylin and eosin.

Quantitative analysis of IL-1β. To assess protein levels, rats were anesthetized before SE and on days 1 (n = 3; 12–24 hours after SE), 7 (n = 3), 14 (n = 3), and 21 (n = 4) after SE. The left hemisphere of each brain was quickly removed, and the hippocampus was dissected on ice and individually homogenized in PBS containing a protease inhibitor cocktail. Homogenates were centrifuged at 4°C, and the supernatants were recovered as samples. IL-1β was measured by using a Bio-Plex Cytokine Assay kit (171-K11070, Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer’s instructions. Briefly, premixed standards were reconstituted by using the provided standard diluents, and a standard curve (0.975–8000 pg/mL) was constructed. An anti-cytokine bead stock solution was added to the wells of a 96-well filter plate. After the filters were washed, the standards and samples (50 μL/well) were added to the wells. Plates were incubated for 30 minutes at room temperature and then washed again, after which 25 μL of detection antibodies was added to each well. After the plates were again incubated and washed as described above, 50 μL of streptavidin–phycoerythrin was added to each well, and the plates were incubated for a further 10 minutes before being similarly washed. Finally, the beads were resuspended in Bio-Plex assay buffer. Plates were read on the Bio-Plex suspension array system (Bio–Rad), and the data were analyzed by using Bio-Plex Manager version 5.0. Statistical analysis was performed with the SPSS 16.0 software (SPSS, Chicago, IL, USA) by using one-way analysis of variance.

Results

Clinical features of seizures and histological findings. Within one hour after KA injection, all rats developed stage 5 seizures according to the Racine scale.21 After the SE was aborted with diazepam, rats developed spontaneous generalized tonic–clonic seizures with a latency of five to seven days.
The histologic changes that occurred after the KA-induced SE were similar to those seen in our previous studies. 3–5 The numbers of pyramidal cells in the control rats were 1083 ± 44, 1183 ± 169, 1150 ± 150, and 983 ± 130 cells/mm² (mean ± SE) in the CA1, CA2, CA3, and CA4 regions of the hippocampus, respectively. The numbers of pyramidal cells on day 7 after SE were 867 ± 109, 808 ± 156, 483 ± 93, and 517 ± 60 cells/mm² in CA1, CA2, CA3, and CA4, respectively. The numbers of pyramidal cells on day 14 after SE were 683 ± 72, 517 ± 93, 500 ± 58, and 433 ± 33 cells/mm² in CA1, CA2, CA3, and CA4, respectively. The numbers of pyramidal cells on day 21 after SE were 133 ± 33, 500 ± 104, 333 ± 93, and 367 ± 44 cells/mm² in CA1, CA2, CA3, and CA4, respectively. Hematoxylin and eosin staining revealed pyknotic pyramidal cells in CA1 on day 1 (Fig. 1B); the degree of pyramidal cell loss in CA1 gradually increased from day 7 to day 21 after SE (Fig. 1C). Pyramidal cell death also occurred in CA3 (rate of loss after SE, 71%) and CA4 (63%) but to a lesser extent than that in CA1 (89%) at day 21. GFAP-immunoreactive astrocytes with subsequent hypertrophy increased from day 7 to day 21 in proportion to pyramidal cell loss, which resulted in hippocampal gliosis (Figs. 1D–H).

**IL-1β immunoreactivity in rat hippocampus during the acute phase after SE.** IL-1β was weakly expressed in the cytoplasm of CA1 and CA3 pyramidal cells in the control rat hippocampus (Figs. 2D, G, I, M). However, in rats with SE, IL-1β expression increased transiently in the cytoplasm of the remaining pyramidal cells in the CA3 region beginning at day 1 after SE (Figs. 2H, N). Cytoplasmic IL-1β expression in pyramidal cells in CA3 was greater than that in CA1 on day 1 after SE (Figs. 2E, H, K, N; Table 1).

**IL-1β immunoreactivity in rat hippocampus during the chronic phase (>7 days) after SE.** At day 7 after SE onset, pyramidal cells in CA1 lacked IL-1β immunoreactivity. However, those in CA3 were still positive for IL-1β. In contrast, reactive astrocyte-like cells emerging in CA1

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**Figure 1.** Hematoxylin and eosin staining and GFAP staining in the rat hippocampus. (A–C) Hematoxylin and eosin staining in the CA1 region of control rats without SE and KA-treated rats after SE. (A) CA1 region at control rat hippocampus. Pyramidal cells were not degenerated. (B) CA1 region on day 1 after SE. Pyknotic pyramidal cells were seen in the CA1 region of rat hippocampus. (C) CA1 region on day 21 after SE. Most pyramidal cells were lost. (D–H) GFAP staining in the CA1 region of control rats without SE and KA-treated rats after SE. (D) CA1 region at control rat hippocampus. A few GFAP positive cells were observed. (E) CA1 region on day 1 after SE. GFAP positive cells on day 1 after SE have no change compared to control rats (D). (F) CA1 region on day 7 after SE. Increased numbers of astroglia-like cells were observed. (G) CA1 region on day 14 after SE. The number of astroglia-like cells in (H) CA1 region on day 21 after SE. GFAP-immunoreactive astroglia-like cells were increased in proportion to the progressive hypertrophy of astrocytes, forming gliosis (G, H).
Figure 2. IL-1β immunoreactivity in the hippocampus of control rat without SE and after SE. (A, D, G, J, M) Expression of IL-1β at control rat hippocampus. (A) Expression of IL-1β at the hippocampus of control rat without SE in low-power magnification. Scale bar = 1 mm. (D) CA1 region of control rat in intermediate power magnification. (G) CA3 region of control rat in intermediate power magnification. D, G; scale bar = 100 μm. (J) CA1 region of control rat in high-power magnification. (M) CA3 region of control rat in high-power magnification. J, M; scale bar = 50 μm. Expression of IL-1β was faintly observed in the pyramidal cells at CA1 (D, J) and CA3 (G, M) regions. (B, E, H, K, N) Expression of IL-1β at day 1 after SE. (B) Expression of IL-1β at the hippocampus at day 1 after SE in low power magnification. Scale bar = 1 mm. (E) CA1 region at day 1 after SE in intermediate power magnification. (H) CA3 region at day 1 after SE in intermediate power magnification. E, H; scale bar = 100 μm. (K) CA1 region at day 1 in high power magnification. (N) CA3 region at day 1 after SE in high power magnification. IL-1β expression increased transiently in the cytoplasm of the remaining pyramidal cells in the CA3 region beginning on day 1 after SE (H, N). Expression of IL-1β in the cytoplasm of pyramidal cells at CA3 was greater than that at CA1 on day 1 after SE (E, H, K, N; Table 1). K, N; scale bar = 50 μm. (C, F, I, L, O) Expression of IL-1β at day 21 after SE. (B) Expression of IL-1β at the hippocampus at day 21 after SE in low-power magnification. Scale bar = 1 mm. (E) CA1 region at day 21 after SE in intermediate power magnification. (H) CA3 region at day 21 after SE in intermediate power magnification. F, I; scale bar = 100 μm. (F) CA1 region at day 21 in high power magnification. (N) CA3 region at day 21 after SE in high power magnification. Reactive astrocyte-like cells emerging in CA1 showed IL-1β immunoreactivity on day 7. This immunointensity increased in proportion to progressive hypertrophy until day 21. L, O; scale bar = 50 μm.
showed IL-1β immunoreactivity at day 7 after SE. Immuno-intensity increased in proportion to progressive hypertrophy until day 21 when the pyramidal cells, which had degenerated because of cell death, lacked IL-1β expression (Figs. 2F, I, L, O; Table 1).

To confirm that the observed IL-1β-immunoreactive astrocyte-like cells were actually reactive astrocytes, we performed double-label immunofluorescence for IL-1β (Fig. 3A) and GFAP (Fig. 3B). Co-localization of IL-1β and GFAP immunoreactivities was confirmed in these reactive astrocytes, which first appeared in CA1 at day 7 and had become numerous at day 21 after SE (Fig. 3C).

Quantitative analysis of IL-1β. Quantitative analysis of IL-1β in control rats without SE (n = 4) and of KA-treated rats on days 1 (n = 3), 7 (n = 3), 14 (n = 3), and 21 (n = 4) after SE onset showed hippocampal IL-1β concentrations of 144 ± 51 pg/mL, 570 ± 180 pg/mL, 2770 ± 798 pg/mL, 1732 ± 344 pg/mL, and 1806 ± 174 pg/mL (mean ± SE), respectively (Fig. 4). At day 1 after SE onset, IL-1β concentration was approximately four times higher in KA-treated rats than in control rats without SE; however, no significant difference in IL-1β concentration in the hippocampus was found between KA-treated rats on day 1 after SE and control rats without SE (P = 0.770). In contrast, in the chronic phase on days 7, 14, and 21 after SE onset, IL-1β concentrations were approximately 20, 12, and 12 times higher, respectively, in KA-treated rats than in control rats without SE. Statistically, the hippocampal IL-1β concentration in KA-treated rats was significantly higher on day 7 (P = 0.003), day 14 (P = 0.029), and day 21 (P = 0.017) than that in control rats without SE.

Discussion
IL-1β is a key pro-inflammatory cytokine in the formation of hippocampal sclerosis after SE; therefore, understanding which cells express IL-1β and the dynamics of IL-1β expression in the hippocampus after SE is important for establishing future therapeutic strategies. Here we elucidated the detailed dynamics of IL-1β expression in the hippocampus. A previous study found that in control rats there is only slight expression of IL-1β in the CA3 region of the hippocampus; however, a different study demonstrated prominent IL-1β expression in scattered neurons of the dentate gyrus and less expression in the neurons in CA3 and CA1.9 Other studies have failed to detect any IL-1β immunoreactivity at all.20,22 In the present study, IL-1β was weakly expressed in pyramidal neurons in CA3, showing that IL-1β is not highly expressed in the hippocampus under normal circumstances in non-epileptic animals. Furthermore, our immunohistochemical and quantitative investigations clearly demonstrated that IL-1β is involved in hippocampal degeneration after SE and that there are differences in IL-1β expression between the acute and chronic phases.

A previous study has shown that three hours after KA administration, IL-1β is expressed on the stratum oriens and

Table 1. Expression of IL-1β in the rat hippocampus.

| HIPPOCAMPAL REGION | CONTROL | 1 DAY AFTER SE | 7 DAY AFTER SE | 14 DAY AFTER SE | 21 DAY AFTER SE |
|---------------------|---------|----------------|----------------|----------------|----------------|
| Pyramidal cells     |         |                |                |                |                |
| CA1                 | +       | +              | +              | −              | −              |
| CA3                 | +       | ++             | +              | −              | −              |
| Reactive astrocytes  |         |                |                |                |                |
| CA1                 | −       | −              | +              | ++             | +++            |
| CA3                 | −       | −              | +              | ++             | ++             |

Notes: Degree of IL-1β-immunoreactive cells: −, no staining; +, faint; ++, frequent; +++ predominant.

Figure 3. Double-label immunofluorescence staining of IL-1β and GFAP. Co-localization of IL-1β (red, A) and GFAP (green, B) is shown with an immunofluorescence method. Co-localization is visualized in yellow in the merged image (C). Double-label fluorescent immunohistochemistry clarified that reactive astrocytes expressed IL-1β. Scale bar = 50 μm.
stratum radiatum in CA1, and the stratum oriens, stratum radiatum, and stratum lucidum in CA3. Other reports have stated that IL-1β immunoreactivity is present throughout the hippocampus 24 hours after KA administration and 48 hours after SE onset. IL-1β is also expressed on microglial cells in the granule and molecular cell layers and hilus of the dentate gyrus within 24 hours after seizures begin. In the present study, IL-1β was expressed on pyramidal neurons in the CA3 region at one and three days after SE. Although the cellular location of IL-1β in our study during the acute phase was not completely consistent with previous studies, IL-1β expression is likely similarly expressed in the pyramidal neurons during the acute phase after SE.

At 60 days after SE, IL-1β is expressed in CA3 by pyramidal neurons and interneurons close to the inner border of the granule cells of the dentate gyrus. Reactive astrocytes prominently express IL-1β, and some microglia in CA3 express IL-1β at four weeks after SE onset. Another report has demonstrated IL-1β immunoreactivity in the CA3 glia at 7 days, but not 60 days, after SE. In the present study, we noted IL-1β expression on reactive astrocytes similar to that shown by previous studies. Although our current findings basically support the expression pattern of pro-inflammatory cytokines that has been previously elucidated, they make several additional contributions. Specifically, immunohistologic methods revealed changes in the localization of IL-1β immunoreactivity across time as it moved from pyramidal cells to reactive astrocytes. In addition, we qualitatively analyzed IL-1β levels in the acute through chronic phase after SE onset. By combining the results of previous studies with the present results, the conclusion that reactive astrocytes are important as the main cells expressing IL-1β during the chronic phase after SE is further strengthened. Because reactive astrocytes are components of hippocampal gliosis, IL-1β can be considered a key factor in glial formation.

Another important finding is that the localization of IL-1β immunoreactivity changes over time and moves from pyramidal cells to reactive astrocytes. Our previous study showed that the survival rate of pyramidal cells in CA1 was 77% at day 7 after SE but only 12% at day 21, and that the survival of these cells in CA3 was 43% at day 7 compared with 29% at day 21. Regardless of the decrease in pyramidal cell number, our present microbead-based assay demonstrated that the total level of IL-1β expression in the hippocampus increased significantly between days 7 and 21 after SE. Simultaneously, the rats developed spontaneous seizures, and reactive astrocytes appeared in the hippocampus at day 7. These findings indicate a transition in the production of IL-1β from pyramidal cells during the acute phase to reactive astrocytes during the chronic phase after SE onset.

Regarding the role of reactive astrocytes, various reports have addressed the pathomechanism underlying the role of reactive astrocytes in epileptogenesis. Glutamine synthetase, which converts glutamate released at excitatory synapses to glutamine, is predominantly expressed in astrocytes. Loss of glutamine synthetase is particularly pronounced in areas of the human mesial TLE hippocampus with astroglial proliferation. In addition, several recent studies have reported that adenosine kinase (ADK) in reactive astrocytes affects seizure development. Upregulation of ADK in astrocytes...
Gial expression of IL-1β in the hippocampus

has been noted in cases of experimental and human TLE. IL-1β and lipopolysaccharide both increase the expression of ADK in cultured human astrocytes, as assessed by western blot analysis. In mice, ADK is a marker for epileptogenesis. Furthermore, adenosine acts as an antiinflammatory that has a neuroprotective role via the adenosine A1 receptor. Another study reported that a complex mixture of signal molecules is released into the extracellular milieu of high-mobility group box 1 (HMGB1)-stimulated astrocytes and that this mixture is functionally involved in the stimulation of monocyte chemotaxis. Human astrocyte cultures obtained during surgery for malformation of cortical development have shown that nuclear to cytoplasmic translocation of HMGB1 is induced by IL-1β. In our present study, IL-1β was expressed on reactive astrocytes, and not pyramidal cells, of the hippocampus during the chronic phase after SE, suggesting an IL-1β-associated role of reactive astrocytes in epileptogenesis during this stage.

In regard to the neuroexcitatory mechanism of IL-1β, N-methyl-D-aspartate receptor function is enhanced by IL-1β through the activation of tyrosine kinases and subsequent phosphorylation of the NR2A/B subunit. This implies that IL-1β contributes to glutamate-mediated neurodegeneration. Other research has indicated that IL-1β affects astrocytes by inhibiting the astrocytic reuptake of glutamate. In addition, IL-1β can increase neuronal glutamate release via the activation of inducible nitric oxide synthase in astrocytes. Together, these studies indicate that inflammatory cytokines chronically influence epileptogenesis. Examination of the time course of the expression and distribution of inflammatory cytokines after SE onset is crucial, and in this regard, our current findings support the results of previous studies.

TLE with hippocampal gliosis is often refractory to treatment. Recent clinical studies have demonstrated increased expression of pro-inflammatory molecules in the neurons and glia of brain tissue obtained from patients treated surgically for drug-resistant epilepsy. Excessive levels of pro-inflammatory cytokines destroy neurons and may lead to seizures. In the context of recurrent seizures, our results suggest that the repeated seizures promoted by IL-1β may expand the epileptic focus and render seizures intractable to therapy.

In conclusion, our study characterized the time course of IL-1β expression in the hippocampus after SE in the acute and chronic phases by using immunohistochemical and quantitative methods. Our results indicated that IL-1β affects the rat hippocampus after SE, especially during the chronic phase. In the acute phase, the main cells expressing IL-1β were pyramidal cells in CA3, whereas in the chronic phase, the main cells expressing IL-1β were reactive astrocytes in CA1.

Author Contributions
Conceived and designed the experiments: SS, TY. Analyzed the data: SS. Wrote the first draft of the manuscript: SS. Contributed to the writing of the manuscript: DT, TY. Agree with manuscript results and conclusions: SS, DT, HO, TY, HS. Jointly developed the structure and arguments for the paper: DT, HO, TY, HS. Made critical revisions and approved final version: SS, DT, HS. All authors reviewed and approved of the final manuscript.

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