Differential Immunoreactivity of Microglial and Astrocytic Marker Protein in the Hippocampus of the Seizure Resistant and Sensitive Gerbils

Choong Hyun LEE1), In Koo HWANG2), In Se LEE2), Ki-Yeon YOO1), Jung Hoon CHOI1), Bong-Hee LEE3)* and Moo-Ho WON1)*

1)Department of Anatomy and Neurobiology, and Institute of Neurodegeneration and Neuroregeneration, College of Medicine, Hallym University, Chuncheon 200–702, 2)Department of Anatomy and Cell Biology, College of Veterinary Medicine and BK21 Program for Veterinary Science, Seoul National University, Seoul 151–742 and 3)Institute for Systems Medicine, Gil Medical Center, Gachon University of Medicine and Science, Incheon 406–799, South Korea

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ABSTRACT. In the present study, we compared differences in ionized calcium-binding adapter molecule 1 (Iba-1) and glial fibrillary acidic protein (GFAP) immunoreactivities for microglia and astrocytes, respectively, in the hippocampus of the seizure-resistant (SR) and seizure-sensitive (SS) gerbils. The density of Iba-1 immunoreactive microglia in the hippocampal CA1 region (CA1) and dentate gyrus (DG) of the SS gerbil was higher than that in the SR gerbil, and many Iba-1 immunoreactive microglia in the SS gerbil were hypertrophied in morphology. In contrast, we could not find significant difference in the density of GFAP immunoreactive astrocytes between the SR and SS gerbils. This result indicates that Iba-1 immunoreactive microglia in CA1 and DG of the SS gerbil are activated compared to those in the SR gerbil.

KEY WORDS: glia, hippocampus, seizure.

Seizure and epilepsy are represented through complex changes, and the consequences of them include excitotoxic cell death and the reorganization of various populations of cells in the central nervous system including the hippocampus [12, 15, 27]. The pathological alterations that occur in the hippocampus following seizures begin within hours and cause changes that last throughout life [13]. It has been also known that neuronal cell death and reactive gliosis occur in distinct but overlapping manner [9, 18, 20].

Microglia are known as the principal immune cells and resident macrophages of the brain, and they have an important role in the defense of the brain against pathological events in the central nervous system. The phagocytosis of the neuronal debris by microglia is well known. It has been reported that microglia participate in repairing and regenerating damaged neurons and in causing neuronal death or dysfunction [1, 2, 21]. Microglia are divided into 5 types according to morphological features, which are amoeboid, ramified (resting), intermediate (hyper-ramified), activated (hypertrophied) and reactive microglia, depending on functional and developmental states [6, 16, 30]. A number of studies have been reported to show the morphological and functional changes of microglia from the resting state to the activated state in response to various neural environments [10, 28].

Astrocytes, one class of glial cells, play important roles in the central nervous system, such as the regulation of ion homeostasis, neurotransmission and neuronal functions [11]. It has been known that astrocytes can promote synapse formation, and maintain and modulate synaptic function [25]. The activation of astrocytes is known to associate with local tissue damage and neuronal loss [14, 29]. Astrocytes are also known to communicate with microglia, and this interaction comes to the front in pathological conditions [33, 35].

Among the number of epilepsy models, Mongolian gerbil is a good model of inherited epilepsy and exhibits spontaneous tonic-clonic motor seizures in response to a variety of stimuli [5, 17]. However, there are few studies about the glial cells in the hippocampus between the seizure sensitive gerbil (SS) and the seizure resistant (SR) gerbil. Therefore, the aim of this study was to investigate differences in immunoreactivities in ionized calcium-binding adapter molecule 1 (Iba-1) for microglia and glial fibrillary acidic protein (GFAP) for astrocytes in the hippocampus between SS and SR gerbils.

The present study used the progeny of Mongolian gerbils (Meriones unguiculatus) obtained from the Experimental Animal Center, Hallym University, Chuncheon, South Korea. Male Mongolian gerbils were used at 3 months (B.W. 65–75 g) of age. The animals were housed in a conventional state under adequate temperature (23°C) and humidity (60%) control with a 12-hr light/12-hr dark cycle, and provided with free access to food and water. The procedures for handling and caring for animals adhered to the guidelines that are in compliance with the current international laws and policies (NIH Guide for the Care and Use of Laboratory Animals, NIH Publication No. 85–23, 1985, 1985).
revised 1996), and they were approved by the Institutional Animal Care and Use Committee (IACUC) at Hallym's Medical Center. All of the experiments were conducted to minimize the number of animals used and the suffering caused by the procedures used in the present study.

Ten seizure sensitive (SS) and ten seizure resistant (SR) gerbils were used in the present experiment. Each animal was stimulated by vigorous stroking on the back with a pencil, as described by Paul et al. [24] and tested a minimum of five times to evaluate the seizure severity of gerbils. According to the seizure severity rating scale of Loskota et al. [17], only animals with a consistent stage 4 or 5 seizure score were included in the present study as SS gerbils. SR gerbils did not demonstrate the seizure activity, namely stage 0. SS gerbils, which had shown severe seizure activity before at least 72 hr, were used prior to next seizure activity.

The animals were anesthetized with a mixture of ketamine (50 mg/kg) and xylazine (10 mg/kg) and perfused via ascending aorta with 0.1 M phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde in 0.1 M PBS (pH 7.4). The brain was removed, post-fixed in the same fixative for 4 hr and cryoprotected by infiltration with 30% sucrose at 4°C for 48 hr. The brain tissues were then frozen and sectioned with a cryostat at 30 µm, and consecutive sections were collected in six-well plates containing 0.1 M PBS.

To ensure that immunohistochemical data were comparable between groups, the free-floating sections were carefully processed under the same conditions. The sections were sequentially treated with 0.3% hydrogen peroxide (H₂O₂) in PBS for 30 min and 10% normal goat serum in 0.05 M PBS for 30 min. They were next incubated with diluted mouse anti-NeuN (neuron-specific soluble nuclear antigen, diluted 1:1,000, Chemicon, Temecula, CA, U.S.A.), rabbit anti-GFAP (diluted 1:1,000, Chemicon) for astrocytes or rabbit anti-Iba-1 (diluted 1:500, Wako, Osaka, Japan) for microglia overnight at room temperature and subsequently exposed to biotinylated goat anti-rabbit IgG (Vector, Burlingame, CA, U.S.A.) for Iba-1/GFAP or goat anti-mouse IgG (Vector) for NeuN and streptavidin peroxidase complex (diluted 1:200, Vector). Then, the sections were visualized by staining with 3,3'-diaminobenzidine in 0.1 M Tris-HCl buffer (pH 7.2) and mounted on gelatin-coated slides. In order to establish the specificity of the immunostaining, a negative control test was carried out with pre-immune serum instead of primary antibody. The negative control resulted in the absence of immunoreactivity in all structures.

Twenty sections per animal were selected in order to quantitatively analyze the density of Iba-1 or GFAP immunoreactive structures. The corresponding areas of the hippocampal CA1 region and dentate gyrus were measured on the monitor at a magnification of 25–50×. Images of all Iba-1 or GFAP immunoreactive structures taken from 3 layers (strata oriens, pyramidale and radiatum in the CA1 region, and molecular, granule cell and polymorphic layers in the dentate gyrus) were obtained through an AxioM1 light microscope (Carl Zeiss, Gottingen, Germany) equipped with a digital camera (AxioCam, Carl Zeiss) connected to a PC monitor. Video images were digitized into an array of 512 × 512 pixels corresponding to a tissue area of 140 × 140 µm (40 × primary magnification). Each pixel res-
The density of immunoreactive structures was evaluated by means of a relative optical density (ROD), which was obtained after the transformation of the mean gray level using the formula: ROD = log (256/mean gray level). ROD of background was determined in unlabeled portions and the value subtracted for correction, yielding high ROD values in the presence of preserved structures and low values after structural loss using NIH Image 1.59 software. A ratio of the ROD was calibrated as %.

The data shown here represent the means ± SEM of experiments performed for each hippocampus. Differences among the means were statistically analyzed by two-tailed student t-test analysis of variance to elucidate differences between SR and SS groups. Statistical significance was considered at P<0.01.

In this study, neurons in the hippocampus of the SR and SS gerbils were observed by NeuN immunohistochemistry. There was no difference in morphology and the number of NeuN immunoreactive neurons in the CA1 region and dentate gyrus in the SS gerbil compared to those in the SR gerbil (Figs. 1 and 4).

In this study, Iba-1 immunoreactive microglia in the SR gerbil were detected in all layers of the CA1 region and dentate gyrus (Fig. 2A and 2B). Most of Iba-1 immunoreactive microglia were ramified (ramified) or intermediate (hyper-ramified) type. In the SS gerbil, the density of Iba-1 immunoreactive microglia in the CA1 region and dentate gyrus of the SS gerbils was much higher than that of the SR gerbil (Figs. 2C, 2D and 4). In addition, many Iba-1 immunoreactive microglia were hypertrophied in morphology (Fig. 2C and 2D).

In the present study, GFAP immunoreactive astrocytes were detected in all layers in the CA1 region and dentate gyrus of the SR gerbil: GFAP immunoreactive astrocytes in the SR gerbil were fewer in the stratum pyramidale of the CA1 region and in the granule cell layer of the dentate gyrus than in the other layers (Figs. 3A and 3B). The morphology and density of GFAP immunoreactive astrocytes in the SS gerbil CA1 region and dentate gyrus were similar to those in the SR gerbil (Figs. 3C, 3D and 4).

It has been known that seizure activity can induce reactive changes of microglia [32, 34]. The precise mechanism of the activation of microglia due to seizures has not been elucidated. However, microglia is known to respond to seizure with altered morphology, gene expression, and proliferation [23, 26, 31]. Unlike astrocytes, the effects of microglial-secreted factors can be neurotoxic in vitro [8].

Drage et al. [7] reported that Iba-1 immunostained microglia in the hippocampus of the epileptic EL mouse, a natural model of idiopathic epilepsy and complex partial seizures, were not amoeboid in morphology, were not amoeboid in morphology, but were similar to the hyper-ramified microglia. The hyper-ramified microglia have been thought to be intermediate state to the resting and reactive states [28]. In addition, Drage et al. [7] confirmed that Iba-1 immunostaining scores were also significantly higher in EL mice rather than in the non-epileptic control.
mice. They also urged that glial abnormalities contributed to the recurrent seizure activity in EL mice. Recently, it has been suggested that microglia are activated even at the resting state, and continuously survey the local microenvironment [22]. Therefore, it needs to examine whether the hypertrophied Iba-1 immunoreactive microglia in the SS gerbil hippocampus are activated or not.

Reactive change in astrocytes in the hippocampus, which means an increase of size and number, is associated with temporal lobe epilepsy in humans and with seizure in animal models [3, 18, 19]. Brigande et al. [4] showed that the number of GFAP immunopositive astrocytes and the content of GFAP protein were significantly greater in the hippocampus of epileptic EL mice than in that of non-epileptic control C57BL/6J mice and that no increase of GFAP immunopositive astrocytes in the EL mice was examined prior to the onset of seizures.

In brief, we found a significant increase in the density of
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Iba-1 immunoreactive microglia in the CA1 region and dentate gyrus of the SS gerbil compared to the SR gerbil, although there was no significant change in GFAP immunoreactive astrocytes in the SS gerbil. These results suggest that increased Iba-1 immunoreactive microglia in the SS gerbil may be related with the environment of the SS gerbil hippocampus.

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REFERENCES