

Age-Related Changes in Ionized Calcium-Binding Adapter Molecule 1 Immunoreactivity and Protein Level in the Gerbil Hippocampal CA1 Region

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ABSTRACT. Microglia are evenly distributed throughout the brain parenchyma. They respond rapidly to a variety of alterations in the microenvironment of the brain and act as sensors for pathological events in the brain. In the present study, we investigated the age-dependent changes in the immunoreactivity and protein level of ionized calcium-binding adapter molecule 1 (Iba-1), a microglial marker, in the CA1 region of the gerbil hippocampus. Iba-1 immunoreactive microglia were detected in the hippocampal CA1 region of the postnatal month 1 (PM 1) group. Iba-1 positive microglia were morphologically inactive between the PM 1 and PM 12 stages. Some Iba-1 immunoreactive microglia were present in the active form in the hippocampal CA1 region of the PM 18 and PM 24 groups. The Iba-1 protein levels in hippocampal CA1 homogenates were decreased in the PM 1 through PM 6 groups and increased in an age-dependent manner thereafter. These results suggest that Iba-1 immunoreactive microglia in the active form were detected in the hippocampal CA1 region in the PM 18 and PM 24 groups. This result may be associated with an age-dependent susceptibility to neurodegenerative diseases associated with the hippocampus.

KEY WORDS: age, gerbil, hippocampal CA1 region, ionized calcium-binding adapter molecule 1, microglia.

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Microglia are a group of small-sized cells of mesodermal origin that act as highly ramified immune sentinels in the brain. They are evenly distributed throughout the brain parenchyma, respond rapidly to a variety of alterations in the microenvironment of the brain, and act as a sensor for pathological events in the brain [18]. Inflammation appears to play a central role among the various processes that have been associated with brain aging.

In the previous studies, microglia were classified into several morphological features; ameboid microglia, intermediate microglia, ramified microglia (resting microglia), activated microglia and phagocytic microglia, depending on their functional and developmental states [6, 20, 27, 34, 35]. During the early postnatal development, the ameboid microglia migrate and proliferate in the brain. Then, they are transformed into the intermediate microglial cell type with elongated process or pseudopodia. Around the second week of postnatal development stage, intermediate microglia decrease, and ramified microglia increase in the rat [6, 20, 34]. Ramified microglia as known resting microglia have a small oval soma with numerous and branched processes. These are spread throughout the entire brain and play an important role in brain homeostasis under the normal condition.

Ramified microglia are transformed into activated microglia and phagocytic microglia after some pathological

events following with inflammatory in the brain. Activated microglia have the significant hypertrophy of cytoplasm with bulbous swelling processes, while phagocytic microglia have a small spherical soma with lack ramified processes [27, 35]. It has been reported that activated microglia contribute to several neurodegenerative diseases via the production of various cytotoxic molecules, free radicals, proinflammatory prostaglandins and cytokines [9, 18, 37, 41].

Ionized calcium-binding adapter molecule 1 (Iba-1) is a novel calcium-binding protein that is specifically expressed in microglia in the brain, which suggests that Iba-1 plays an important role in regulating the function of microglia [11, 14, 16, 17]. Recent studies have reported Iba-1, also known as allograft inflammatory factor-1 (Aif1), in regulating the rearrangement of the actin cytoskeleton [25]. The expression of Iba-1 is up-regulated in microglia following brain injuries and diseases. Therefore, Iba-1 is utilized as a microglial marker in several studies [7, 9, 16, 25].

Mongolian gerbils are good animal models of aging, stroke, epilepsy and some stress experiments because of their brain vasculature, genetic characteristics and relatively short lifespan [12, 30, 43, 44]. Although many recent studies have reported a significant age-related increase in the number of activated microglia and astrocytes in mice, rats, nonhuman primates, and humans [24, 31, 32], there are no studies on the age-related changes in Iba-1 in the gerbil hippocampal CA1 region. Therefore, in this study, we investigated Iba-1 immunoreactivity and protein level in the gerbil hippocampal CA1 region at various age stages.

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MATERIALS AND METHODS

Experimental animals: Male Mongolian gerbils (*Meriones unguiculatus*) were obtained from the Experimental Animal Center, Hallym University, Chuncheon, South Korea. The animals were kindly supplied by Dr Oh, a chief in the Experimental Animal Center at Hallym University. Postnatal month (PM) 1 (n=12), PM 3 (n=12), PM 6 (n=12), PM 12 (n=12), PM 18 (n=12) and PM 24 (n=12) gerbils 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 free access to food and water. The procedures for handling and caring for the animals adhered to the guidelines [AH1] 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, revised 1996). 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.

Tissue processing: For the histological analysis, seven animals per each group were anesthetized with sodium pentobarbital and were perfused transcardially with 0.1 M phosphate-buffered saline (PBS, pH 7.4), followed by 4% paraformaldehyde in 0.1 M phosphate-buffer (PB, pH 7.4). The brains were removed and postfixed in the same fixative for 6 hr. The brain tissues were cryoprotected by infiltration with 30% sucrose overnight. Thereafter, frozen tissues were serially sectioned on a cryostat (Leica, Germany) into 30- μ m coronal sections, and the sections were then collected into six-well plates containing PBS.

Immunohistochemistry for Iba-1: Immunohistochemistry was performed under the same conditions in gerbils of different ages in order to examine whether the degree of immunohistochemical staining was accurate. 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 then incubated with diluted rabbit anti-Iba-1 (Wako, 1:500, Japan) overnight at room temperature and subsequently exposed to biotinylated goat anti-rabbit IgG and streptavidin peroxidase complex (diluted 1:200, Vector, U.S.A.). They were then visualized by staining with 3,3'-diaminobenzidine (Sigma, St. Louis, MO, U.S.A.) in 0.1 M Tris-HCl buffer (pH 7.2) and mounted on gelatin-coated slides. The sections were mounted in Canada Balsam (Kato, Japan) following dehydration. A negative control test was carried out using pre-immune serum instead of primary antibody in order to establish the specificity of the immunostaining. The negative control resulted in the absence of immunoreactivity in any structures. We referred to previous studies to distinguish and explain the morphological characteristics of microglia observed in this work [6, 20, 27, 34].

Western blot analysis: The experimental animals (n=5 per each group) were sacrificed and used for Western blot analysis in order to obtain accurate data for analyzing the changes in Iba-1 in the hippocampal CA1 region at various

ages. After the hippocampus was removed from each animal, it was transversely cut into a thickness of 400 μ m on a vibratome (Leica, Germany), and the hippocampal CA1 region was then dissected with a surgical blade. The tissues were homogenized in 50 mM PBS (pH 7.4) containing EGTA (pH 8.0), 0.2% NP-40, 10 mM EDTA (pH 8.0), 15 mM sodium pyrophosphate, 100 mM β -glycerophosphate, 50 mM NaF, 150 mM NaCl, 2 mM sodium orthovanadate, 1 mM PMSF and 1 mM DTT. After centrifugation, the protein level in the supernatants was determined using a Micro BCA protein assay kit with bovine serum albumin as a standard (Pierce Chemical, U.S.A.). Aliquots containing 20 μ g of total protein were boiled in loading buffer containing 150 mM Tris (pH 6.8), 3 mM DTT, 6% SDS, 0.3% bromophenol blue and 30% glycerol. The aliquots were then loaded onto a 12.5% polyacrylamide gel. After electrophoresis, the gels were transferred to nitrocellulose transfer membranes (Pall Crop, East Hills, NY, U.S.A.). To reduce background staining, the membranes were incubated with 5% non-fat dry milk in PBS containing 0.1% Tween 20 for 45 min, followed by incubation with rabbit anti-Iba-1 antiserum (1:500), peroxidase-conjugated goat anti-rabbit IgG (Sigma, U.S.A.) and an ECL kit (Pierce Chemical, Rockford, IL, U.S.A.).

Quantification of data and statistical analysis: Fifteen sections per animal were randomly selected from the corresponding areas of the hippocampus in order to quantitatively analyze Iba-1 immunoreactivity in the hippocampal CA1 region.

The corresponding areas of the hippocampal CA1 region were measured on the monitor at a magnification of 25–50 \times . Images of all Iba-1 immunoreactive structures taken from 3 layers (strata oriens, pyramidale and radiatum) were obtained through an AxioM1 microscope (Carl Zeiss, Germany) equipped with a digital camera (Axiocam, Carl Zeiss, Germany) connected to a PC monitor. The images were processed into an array of 512 \times 512 pixels corresponding to a tissue area of 140 \times 140 μ m (40 \times primary magnification). Each pixel resolution was 256 gray levels. The intensity of all Iba-1 immunoreactive structures was evaluated on the basis 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). The ROD of the complete field was measured, and the level of background staining was subtracted from the ROD level of the immunoreactive structure before statistical processing. The relative % of control level was demonstrated in the graph. The results of the Western blot analysis were also scanned, and the ROD was determined using Scion Image software (Scion Corp., U.S.A.).

The data shown here represent the means \pm S.E. of experiments performed for each experimental area. Differences among the means were statistically analyzed by one-way analysis of variance followed by Duncan's new multiple range method or Newman-Keuls test in order to elucidate differences between PM 1 and other aged groups.

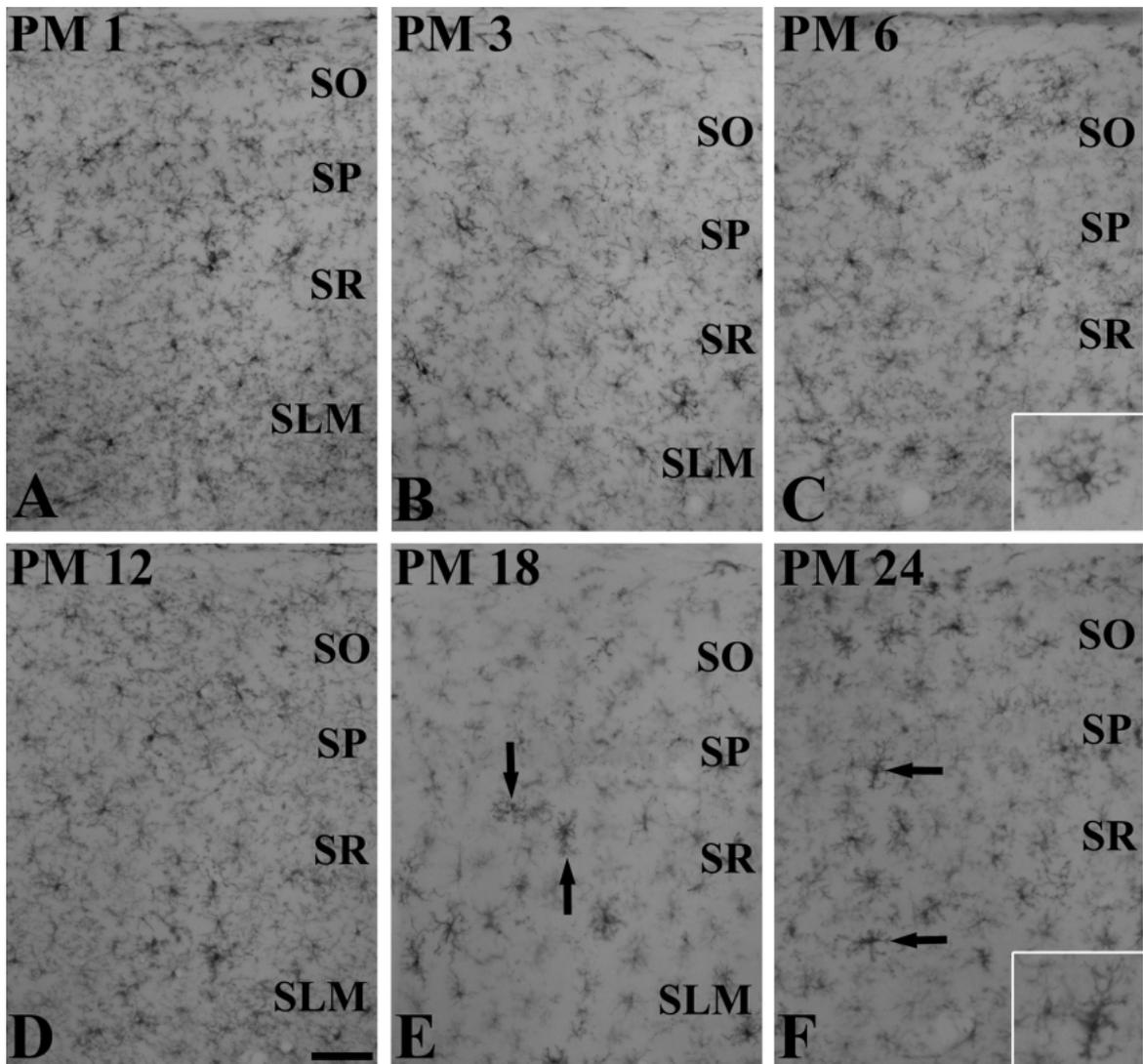


Fig. 1. Immunohistochemical staining for ionized calcium-binding adapter molecule 1 (Iba-1) in the gerbil hippocampal CA1 region at postnatal month (PM) 1 (A), PM 3 (B), PM 6 (C), PM 12 (D), PM 18 (E) and PM 24 (F). Iba-1 immunoreactive microglia are detected in the hippocampal CA1 region of all age groups. Some Iba-1 immunoreactive microglia in the active form (arrows) are detected in the PM 18 and PM 24 groups. Ramified microglia (C) and activated microglia (F) are shown in the small box. Strata oriens (SO), pyramidale (SP), radiatum (SR) and lacunosum moleculare (SLM). Bar = 100 μ m.

RESULTS

Changes in Iba-1 immunoreactivity at various ages: Iba-1 immunoreactivity was detected in stratum oriens (SO), radiatum (SR), pyramidale (SP) as well as stratum lacunosum moleculare (SLM) of the CA1 region in the PM 1 group (Fig. 1A). Especially, Iba-1 immunoreactivity was high in SLM of the hippocampal CA1 region. In this group, the cytoplasm of the Iba-1 immunoreactive cells was small, while their processes were highly ramified. In the PM 3 group, Iba-1 immunoreactive cells were also detected in all layers of the hippocampal CA1 region. The Iba-1 immunoreactivity was slightly decreased in the SLM of hippocampal CA1 region, while in the SO, SR and SP of hippocampal CA1 region, Iba-1 immunoreactivity was higher than that in the PM 1 group (Figs. 1B and 2). In this group, in addition, the cytoplasm of the Iba-1 immunoreactive cells was also small and had well-developed processes (Fig. 1B). Iba-1 immunoreactive cells were also detected in the hippocampal CA1 region in the PM 6 group. In this group, Iba-1 immunoreactivity was decreased in the hippocampal CA1 region (Figs. 1C and 2). Especially, in this group, Iba-1 immunoreactivity was very low in the SLM of hippocampal CA1 region (Fig. 1C). In the PM 1 to PM 6 groups, Iba-1 immunoreactive small cells had well-developed processes, and these cells were identified as ramified

microglia. In the PM 12 group, Iba-1 immunoreactivity was high in all layers of the hippocampal CA1 region (Fig. 1D). In the PM 18 group, Iba-1 immunoreactivity was high in all layers of the hippocampal CA1 region (Fig. 1E). In this group, some Iba-1 immunoreactive microglia in the active form (arrows) were detected in the hippocampal CA1 region (Fig. 1E). In the PM 24 group, Iba-1 immunoreactivity was high in all layers of the hippocampal CA1 region (Fig. 1F). In this group, some Iba-1 immunoreactive microglia in the active form (arrows) were detected in the hippocampal CA1 region (Fig. 1F). In this group, some Iba-1 immunoreactive microglia in the active form (arrows) were detected in the hippocampal CA1 region (Fig. 1F). In this group, some Iba-1 immunoreactive microglia in the active form (arrows) were detected in the hippocampal CA1 region (Fig. 1F).

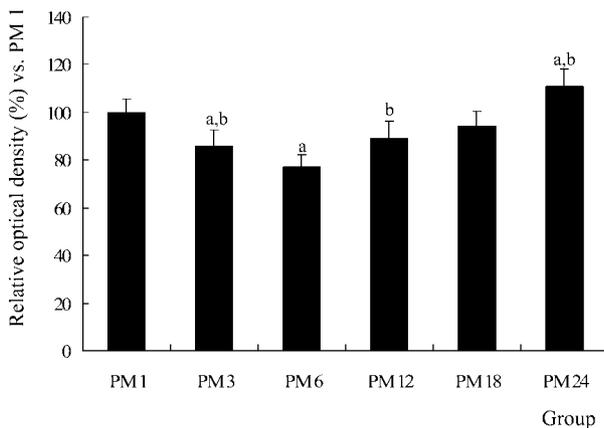


Fig. 2. Relative optical densities of age-induced changes in Iba-1 immunoreactivity in the hippocampal CA1 region. Differences among the means were statistically analyzed by one-way analysis of variance followed by the Duncan's new multiple range method or Newman-Keuls test ($n=7$ per group; ^a $P<0.05$, significantly different from the postnatal month (PM) 1 group, ^b $P<0.05$, significantly different from the pre-adjacent group). The bars indicate the means \pm S.E.

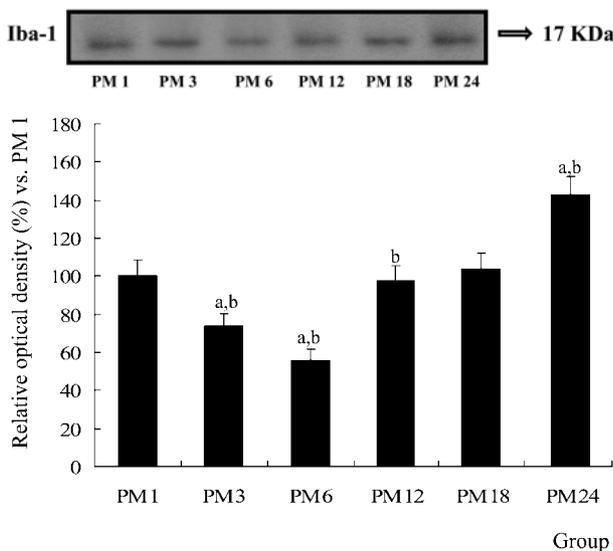


Fig. 3. Western blot analysis of Iba-1 in the gerbil hippocampal CA1 region derived from gerbils in various age groups. The relative optical density of the immunoblot band is also represented ($n=5$ per group; ^a $P<0.05$, significantly different from the postnatal month (PM) 1 group, ^b $P<0.05$, significantly different from the pre-adjacent group). The bars indicate the means \pm S.E.

microglia based on their morphology (Fig. 1A-1C).

In the PM 12 group, most of the Iba-1 immunoreactive cells were also ramified microglia, and their processes were increased in comparison to the PM 6 group (Figs. 1D and 2). Scarcely, Iba-1 immunoreactive cells with swollen processes like activated microglia were detected in the PM 12 group (data not shown). Significant morphological changes

in Iba-1 immunoreactive microglia were observed in the CA1 region through the PM 18 to PM 24 groups. Some Iba-1 immunoreactive cells showed an increased cell body with shortened swollen processes, and some had an elongated cell body (Fig. 1E and 1F). These cells were identified as activated microglia based on their morphology. The number of the activated microglia immunoreactive for Iba-1 in the PM 24 group increased compared to the PM 18 group (Figs. 1F and 2).

Changes in Iba-1 protein level at various ages: Western blot analysis showed that the pattern of Iba-1 expression in the hippocampal CA1 region at various ages was similar to that of the immunohistochemical data in the experimental groups. The Iba-1 protein level decreased in an age-dependent manner in the PM 1 through PM 6 groups. Thereafter, the Iba-1 protein level increased in an age-dependent manner. The Iba-1 protein level in the PM 18 group was similar to that in the PM 1 group, and the highest Iba-1 protein level was detected in the PM 24 group (Fig. 3).

DISCUSSION

The activation of glial cells [24, 33, 35], and increases in cytokines and their receptors [4] were previously reported in the aged brain using histological and gene expression analyses [3, 21, 37].

In the present study, we observed that the morphology and Iba-1 level in Iba-1 immunoreactive microglia were changed in the hippocampal CA1 region with age in gerbils. Iba-1 immunoreactive microglia were abundant in the hippocampal CA1 region of all the age groups. In the morphological feature, most of Iba-1 immunoreactive microglia had a small cell body with well developed processes and distributed through the PM 1 to the PM 12 groups. These microglia were identified as ramified microglia, known as resting microglia that are generally present in the hippocampal CA1 region of the normal brain as well as other brain regions in previous studies [1, 6, 20, 27].

In the PM 1 group, many Iba-1 immunoreactive microglia were observed in the SLM of the hippocampal CA1, while small amount of Iba-1 immunoreactive microglia were observed in the SR relatively. Iba-1 immunoreactive microglia in the PM 3 group decreased in the SLM of the hippocampal CA1, whereas they increased in the SR. It may be related with the migration of microglia from the hippocampal fissure through the SLM to main cell layers (SR, SO, SP) of the CA1 region during the postnatal development.

The Iba-1 protein level showed a decreasing pattern with age through the PM 1 to PM 6 groups. During the prenatal and postnatal development, natural neuronal death is known to be present, and essentially, it is regarded as a process of elimination of excessive number of cells arising from the neuroepithelium by the surrounding glia such as microglia [5, 26, 28]. The ramified microglia possess rich endowment of filament proteins such as actin and tubulin. This filament system in the microglia is highly related to the microglia endocytotic activity such as phagocytosis or pinocytosis

[40]. Therefore, this result may be associated with the ramified microglial functions that were up-regulated during the postnatal development for the elimination of products from natural cell death and axonal elimination. Interestingly, activated microglia immunoreactive for Iba-1 were increased in the PM 18 and 24 groups. The activated microglia showed that cell bodies were increased in size, and processes were thickened in the proximal part and decreased in the ramification of distal branches [19, 29].

In addition, Iba-1 protein level in the PM 24 group was highest among the experimental groups, and activated microglia significantly increased in the PM 12 group. It may be associated with neurodegeneration in the hippocampal CA1 region in gerbils. In the gerbil life span, the mean survival rate (50%) of male gerbil is 110 weeks, and the survival rate of male gerbil is significantly decreased from 40 to 50 weeks, and thereafter decreased with age in nature [39]. There was no direct evidence for decreased death rate related with neurodegeneration in the gerbil, but it may assume that there are ongoing some neurodegeneration with age which can activate the resting microglia. It has been reported that the reduction of extracellular volume, increased gliosis, neuronal death, decline in physical activity and spatial learning deficit were related with age in laboratory rodents [2, 7, 8, 12, 23, 36]. In addition, microglia change their morphological features such as swelling process, cytoplasmic vacuoles and hypertrophy in the aged rat and mouse brain as well as in the Huntington disease mouse model [22, 24]. For these reasons, it has been widely accepted that microglia may contribute to the neurodegeneration through a release of the variety of proinflammatory and potentially neurotoxic substances with age. As mentioned in the introduction, the expression of Iba-1 is upregulated in activated microglia within the regenerating facial nucleus and in the ischemic insult [13, 16, 17]. Also, it has been reported the Iba-1 protein involved in Rac and the calcium signaling pathway is a key molecule in membrane ruffling and the phagocytosis of microglia [25]. Therefore, the results that increased activated microglia and Iba-1 protein level in the aged hippocampal CA 1 region may be associated with age-related neurodegeneration in this region.

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