Different Glial Reactions to Hippocampal Stab Wounds in Young Adult and Aged Rats

Waner Zhu,1 Hiroyuki Umegaki,1 Tadashi Shinkai,2 Shinobu Kurotani,1 Yusuke Suzuki,1 Hidetoshi Endo,3 and Akihisa Iguchi1

1Department of Geriatrics, Nagoya University Graduate School of Medicine, Japan.  
2Department of Cell Biology, Tokyo Metropolitan Institute of Gerontology, Japan.  
3Department of Geriatrics, Chubu National Hospital, Morioka, Japan.

Brain injury induces reactive gliosis. To examine the activation of glial cells after brain injury in young versus aged rats, we used a brain stab-wound model and examined the expression of cells positive for ED1 (ED1⁺) and glial fibrillary acidic protein (GFAP⁺) in the hippocampus in young-mature (3 months) and aged (25 months) Wistar rats at various times following hippocampal stab injury. ED1⁺ cells appeared more frequently in the aged rats than in the young-mature rats under control conditions, whereas the number of GFAP⁺ cells was not different between two groups. Following the stab wound, there was an increase in ED1 expression that was delayed but stronger in the aged rats and that persisted longer; the increase of the number of GFAP⁺ cells also persisted longer. We conclude that different glial reactivity in the aged brain suggests that aging is associated with increased glial responsiveness that may enhance susceptibility to injury and disease in the brain.

MICROGLIA are ubiquitous in the brain and are the main immune effectors of the central nervous system (1,2). Under normal conditions they show a downregulated immunophenotype but can be activated in response to the cytotoxic and inflammatory processes that follow brain injury (3), neurodegenerative diseases including Alzheimer’s disease (4), and the aging process (5).

Activation of microglia is characterized by proliferation and recruitment to the site of injury. Activated microglia can become phagocytic and are then morphologically indistinguishable from macrophages originating from bloodborne monocytes (6). When transformed into macrophages, microglia express a lysosomal monocyte–macrophage antigen, ED1 (7). The phagocytic activity has been confirmed to correlate with the degree of ED1 expression (8), making ED1 a convenient marker for phagocytic microglia. Astrocytes, which are identified by glial fibrillary acidic protein (GFAP) (9), also become activated after injury, inflammation, and some types of infection, and in response to neurodegenerative processes including Alzheimer’s disease (10,11).

The rodent stab-injury model has been extensively utilized for studies of inflammation in the central nervous system, and its effects on brain tissues are well documented (12). Glial cells become activated within 2–3 days following a stab injury, which has been confirmed by expression of ED1 and GFAP immunoreactivity (12,13).

Aging is an established risk factor for increased mortality following traumatic brain injury (14). It was recently reported that, in the cortex, stab injury resulted in an increased induction of ED1 at 72 hours in aged versus young rats (15). However, to our knowledge, age-related changes in glial activation after stab injury have not been examined in the hippocampus. The hippocampus is a structure that is deeply involved in the neuropathology of Alzheimer’s disease, one of the most common age-related neurodegenerative disorders, whose pathogenesis is presumed to involve a chronic inflammatory process as evidenced by glial activation (4). A study showed that brief global ischemia was associated with different glial responses in the cortex and hippocampus (16). Therefore, age-associated changes of glial reactions in the hippocampus may be different from those in the cortex. To investigate this, we studied ED1-positive (ED1⁺) cells and GFAP-positive (GFAP⁺) cells in the hippocampus at various time points after hippocampal stab wounds in young and aged rats.

METHODS

Subjects

Twenty-one young-mature (3 months old; weight 250–300 g) Wistar rats, which were purchased from SLC Inc., Japan, and 21 aged (25 months old; 450–500 g) rats of the same strain, supplied by the Department of Laboratory Animal Science at the Tokyo Metropolitan Institute of Gerontology, were used in this study. Animals were housed individually under standard laboratory conditions in temperature-controlled rooms (24°C), and they were maintained on a 12-hour light–dark cycle (lights on at 6 AM) with food pellets and water available ad libitum. All rats were cared for in accordance with the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources and approved by the Nagoya University Graduate School of Medicine Animal Care and Use Committee.
Surgery
Animals were anesthetized with an intraperitoneal injection of a mixture of ketamine (40 mg/kg) and xylazine (20 mg/kg). The rats were positioned in a stereotaxic apparatus (Narishige Scientific Instrument Laboratory, Tokyo, Japan). A midsagittal incision was made in the scalp to expose the skull, and a burr hole was drilled overlying the needle insertion coordinates. A hippocampal cannula was made by inserting an 23-gauge needle at the following coordinates determined from the atlas by Paxinos and Watson (17): 4.52 mm posterior to bregma, 3 mm lateral to the sagittal suture, and 3.5 mm below the dura.

Immunohistochemistry
For immunohistochemical analysis, the animals were sacrificed at 2, 5, 7, 14, 21, and 28 days after the surgery (n = 3 for each time point). Three young-mature rats and three aged rats who did not undergo the surgery served as controls. Rats were deeply anesthetized with an intraperitoneal injection of a lethal dose of a mixture of ketamine and xylazine and perfused transcardially with saline followed by 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). The removed brains were postfixed overnight and cryoprotected in phosphate-buffered saline (PBS) containing 30% sucrose. Brains were immediately frozen with powdered dry ice. Serial coronal sections (30 μm in thickness) were made throughout the regions of the wounds with a cryostat. Floating sections were then immunostained by the preformed avidin–biotin complex (ABC)–DAB (3,3’-diaminobenzidine) method, as described previously (15). In brief, the sections were repeatedly washed in PBS and incubated overnight at 4°C with a mouse monoclonal antirat antibody to ED1 (dilution 1:500; MCA341R; Serotec Ltd., UK) or a mouse monoclonal antibody to GFAP (dilution 1:1000; MAB3402; CHEMICON International, Temecula, CA). Sections were rinsed in PBS, incubated in ABC (Vectastain Elite Kit, Vector Labs, Burlingame, CA). Sections were rinsed in PBS, incubated in ABC (Vectastain Elite Kit, Vector Labs) for 60 minutes, washed several times in PBS, and then visualized by use of a DAB kit (Vector Labs). Sections were washed again in PBS, mounted on glass slides, air dried, dehydrated, and cover-slipped. Sections from the 14 experimental groups, representing all of the different time points, were processed simultaneously by use of the same solutions.

Semiquantitative Counts and Statistics
All the sections were assessed by means of microscopic examination by using an Olympus BX50 microscope (Tokyo, Japan). Photographs were made of representative ED1+ cells in the wound area. For each time point, 3 animals were used. For each animal, five to six sections in the area containing the wound were examined. Counting of cells stained for ED1 was carried out in three to four sections in the region (0.2925-mm² field) that corresponded to the center of the wound, where the reaction was more prominent. The total number of ED1+ cells was counted and then divided by the total number of sections examined, and the quotient served as a mean cell count per slice for statistical analysis. The number of GFAP+ cells was estimated by the same method as ED1+ cells.

Statistical significance between the two groups (3 months vs 25 months old) was assessed by a repeated-measures one-factor analysis of variance (ANOVA). Student’s t test was used for statistical comparison at each time point between the two groups. A level of p < .05 was accepted as statistically significant.

RESULTS
Photographs depicting hippocampal ED1 immunostaining from selected experimental conditions are displayed in Figure 1. Under control conditions, ED1+ cells appeared more frequently in the aged rats (Figure 1B) than in the young-mature rats (Figure 1A). After the stab wounds were induced, ED1+ cells increased from Day 2 in the young-mature rats (Figure 1C), peaked at Day 5 (Figure 1E), and then gradually decreased until Day 28 (Figure 1G). In contrast, in the aged rats, ED1+ cells increased from Day 5 (Figure 1F), and they remained at a high level until Day 28, the end of the experiment (Figure 1H). The total numbers of ED1+ cells in the ipsilateral hippocampus are presented as means ± SD in Figure 2. A repeated-measures one-factor ANOVA indicated a highly significant difference between the number of ED1+ cells in the two groups (p = .0002), and the magnitude of the increase was greater in the aged rats. Student’s t test indicated a significant difference in the increase of ED1+ cells between the two groups under control conditions (p = .009), and on Days 2 (p = .002), 7 (p = .007), 14 (p = .0006), 21 (p = .0003), and 28 (p = .0001) after the stab wounds. Under control conditions as well as on Days 7, 14, 21, and 28 after the stab wounds, there was a greater number of ED1+ cells in the aged rats than in the young-mature rats, whereas there was a significantly greater increase in the number of ED1+ cells in the young-mature rats than in the aged rats only on Day 2 after the stab wounds.

The total numbers of GFAP+ cells in the ipsilateral hippocampus are presented as means ± SD in Figures 3 and 4. Student’s t test showed no significant age-associated difference between young-mature and aged groups in control conditions (p = .488). Repeated ANOVAs demonstrated a significant age effect on the changes of GFAP+ cells (p = .0016).

DISCUSSION
Several changes in microglia have been noted during the aging process. An increase in the number of activated microglia occurs in several regions of the aged rat brain, but mainly in the white matter (5,18). Furthermore, Sheffield and Berman (19) found a positive correlation between the density of activated microglia in the cerebral white matter and cognitive impairment in aged primates. In the present study, we found that even in the absence of injury, activated microglia appear more frequently in the hippocampi of aged rats than in those of young-mature rats. Aged microglia in
Figure 1. ED1 protein expression is induced in the brain of aged and young-mature rats following hippocampal stab wounds. ED1 immunoreactivity is shown in young-mature (left) and aged (right) rat brains. Before injury, aged animals (B) showed higher expression of ED1 than young-mature rats (A). ED1 immunoreactivity was higher at 2 days (C, D), 5 days (E, F), and 28 days (G, H) after stab injury than at baseline. Aged animals showed delayed but stronger and more persistent ED1 expression than young-mature rats. Bar = 50 μm.
the hippocampus may therefore maintain elevated levels of reactivity even without external stimuli. Reportedly there is an age-associated increase in the number of human cortical astrocytes immunoreactive for GFAP (20). In the current study we observed no age-associated difference in the number of GFAP$^+$ cells in the hippocampus in the basal condition, which was in agreement with the observation in the striatum (21).

Although activation of microglia by cortical stab injury in young-mature and aged rats has been documented in previous studies (12,13,15), we believe that we have shown the effects of aging on the glial response to a hippocampal...
stab injury for the first time, both qualitatively and quantitatively. Our results indicate that induction of activated glia is different in aged rats versus young-mature rats after a hippocampal stab injury. The induction of activated microglia was delayed in the aged rats, but it was stronger and persisted longer. In addition to microglial activation, infiltration of circulating macrophages may also have contributed to increased ED1 immunoreactivity, because both cell types express the ED1 antigen (7,12). Although not examined here, age-related differences in recruitment and infiltration of peripheral monocytes may exist. Kyrkanides and associates showed enhanced glial reactions following cortical stab injury in aged rats (15). They demonstrated that, as early as 6 hours after injury, aged rats had significantly stronger glial reactions indicated by positive staining for ED1 and GFAP than younger rats did. In the current study, however, the number of ED1⁺ cells increased from Day 5 and kept a high level as long as 4 weeks. We also estimated astrocyte reactions by GFAP staining. Although the number of GFAP⁺ cells was similar at Day 5 after stab wounds in young-mature and aged groups, the increase of the number of GFAP⁺ cells persisted longer until Day 21 in the aged group (Figure 3). Astrocytes indicated by GFAP staining demonstrated larger cell bodies and longer thicker processes, which suggested increased activity (Figure 4). Kyrkanides and associates showed that, in the cortex, reaction of astrocytes was enhanced (15), and a study done in the striatum demonstrated no age-associated difference in GFAP reactivity 4 days after stab wounds (21). Taken together, these age-associated changes of glial reactions may be differently expressed in each brain region.

Several mechanisms may be responsible for the different responsiveness of the aged rats to hippocampal stab injury. The induction of ED1⁺ cells in the aged brain after a stab injury may be delayed if the release of gliotrophic factors in response to injury, or the microglial reaction to those factors following injury, becomes slower with age. Injury may also produce larger amounts of damage in the aged brain, or the aged microglia in the hippocampus may have higher reactivity to the stab injury, resulting in a stronger induction of ED1⁺ cells, which is presumably indicative of stronger microglial induction. Persistent expression of the ED1⁺ and GFAP⁺ cells in the aged brain after stab injury may imply that the mechanism for maintaining immunologic homeostasis is impaired in the aged rat brain.

It has been well documented that activated microglia and astrocytes are involved in cytotoxic and inflammatory processes in neurodegenerative diseases as well as during the aging process (1,4,5,11). Reactive microglial cells exist in the center of the senile plaques, which is the hallmark pathology of Alzheimer’s disease (22), while reactive astrocytes form outer shells around them, with fibrils extending into the center (23). Amyloid-β (Aβ), which is a major component of senile plaques, has been shown to activate microglial cells (24,25), and a recent study indicated that microglial cells are the major factor driving plaque formation by fibrillar Aβ deposition (26). Clinical studies have established that mortality rates following traumatic brain injury and stroke are worsened by age (14). Although many physiologic factors may contribute to poor outcomes, our results support the idea that age-related changes in the microglial reaction to injury are likely to play some role in the increasing susceptibility to traumatic brain injury or diseases including neurodegenerative diseases and stroke, which are commonly found during the aging process.

Conclusions

In summary, the present study found that ED1⁺ cells appeared more frequently in aged rats than in young-mature rats under control conditions. Following hippocampal stab wounds, the increased expression of ED1⁺ cells was delayed but stronger in the aged rats, and it persisted longer; the reaction of GFAP⁺ cells also persisted longer. These data suggest that aging is associated with an enhanced glial responsiveness.
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Address correspondence to Hiroyuki Umegaki, PhD, MD, Department of Geriatrics, Nagoya University Graduate School of Medicine, 65 Tsuruma-Cho, Showa-Ku, Nagoya, Aichi, 466-8550, Japan. E-mail: umegaki@tsuru.med.nagoya-u.ac.jp

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