Intracerebral Hemorrhage-Induced Brain Injury Is Aggravated in Senescence-Accelerated Prone Mice

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Background and Purpose—In cerebral stroke, the overall mortality rate of older individuals is higher than that of younger individuals. We therefore investigated aging-related changes in brain tissue damage and immune response in response to intracerebral hemorrhage (ICH) in mice.

Methods—ICH was induced by microinjecting autologous whole blood (5 μL) into the striatum of 4- or 14-month-old senescence-accelerated prone (SAMP8) mice or senescence-accelerated resistant (SAMR1) mice.

Results—In all groups, neurological deficits occurred within 6 hours and gradually improved after the first day, but improvement was most delayed in 14-month-old SAMP8 mice. Isolectin B4-positive and amoeboid microglia/macrophages were abundantly distributed around and inside the hemorrhagic lesions in 14-month-old SAMP8 mice. In contrast, myeloperoxidase-immunoreactive neutrophils and reactive astrocytes with intensified glial fibrillary acidic protein–stained processes and swollen cytoplasm did not differ in number or distribution between SAMP8 and SAMR1 mice. Regardless of their age, the immunoreactivity of Mn-SOD, a major antioxidant enzyme in mitochondria, was much weaker in SAMP8 than in SAMR1 mice. The expression of inducible nitric oxide, however, was higher in old SAMP8 mice than in the other experimental groups.

Conclusions—These results suggest that activated microglia/monocytes may aggravate intracerebral hemorrhagic damage in old SAMP8 mice. Further studies on the exact role of activated microglia/monocytes and the altered activities of antioxidant enzymes in old SAMP8 mice may provide useful information for ICH-induced brain injury in relation with aging. (Stroke. 2006;37:216-222.)

Key Words: aging ■ cerebral hemorrhage ■ mice ■ microglia ■ monocytes ■ oxidative stress

Cerebral stroke is the second leading cause of death worldwide, and its incidence is expected to rise with the projected aging of the population. Most clinical cases are associated with hypertension.1 During an intracerebral hemorrhage (ICH), blood rushes into the surrounding brain tissue at high pressure, resulting in damage to brain tissue. Neurologic impairments result from direct tissue destruction, compression of the surrounding tissue, and cerebral edema.2 ICH commonly occurs in the striatum, pons, thalamus, and cerebellum, and accounts for ≈10% of all strokes.1,3,4 Mortality is ≈50% within 30 days, and only 10% of survivors can live independently.5 At present, however, the pathogenesis of damage after ICH is not well understood.

In general, cerebral hemorrhage occurs more frequently in the elderly than in the young. Previous studies in rats have shown that, during cerebral stroke, the overall mortality rate was much higher in old rats than in young rats.6 Little is known about the effect of aging on hemorrhagic brain damage, although hippocampal tissue from old animals was recently reported to be more susceptible to ischemia-reoxygenation injury.7 Furthermore, ICH caused greater brain edema and neurological deficits in aged than in younger rats,8 suggesting that aging may have a profound effect on ICH-induced brain injury.

Senescence-accelerated mouse (SAM) strains have been established as murine models for accelerated aging.9,10 To date, 9 senescence-prone (SAMP) and 4 senescence-resistant (SAMR) strains have been developed, each of which shows various age-associated and strain-specific pathologic phenotypes, such as shortened life-span and early manifestation of senescence.11 Among the SAM sub-strains, the SAM prone 8 (SAMP8) strain exhibits deficits of learning and memory,12 impaired immune response,13 and abnormal circadian rhythms.14 In the present study, therefore, we investigated...
aging-related changes in brain tissue damage and immune response after ICH in SAMP8 mice and in control SAMR1 mice.

Materials and Methods

Animals
Pathogen-free SAMP8 and SAMR1 mice (male; body weight ∼30 g regardless of age) were kindly donated by Dr. Yong-Sun Kim (Hallym University, South Korea) and housed under conditions of controlled temperature (23±1°C) and humidity (55±2%). Animals had access to mouse chow and water ad libitum and were used for experiments at 4 or 14 months of age. All experimental procedures using animals were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Committee of Ewha Women’s University College of Medicine.

Autologous Whole Blood–Induced ICH
Mice were anesthetized with chloral hydrate (300 mg/kg, IP; Sigma) and placed in a stereotaxic frame (David Kopf Instruments). Through a hole drilled in the skull, a 32-gauge needle was implanted into the striatum 2.0 mm lateral to the midline, 1 mm anterior to the coronal suture, and at a depth of 3.8 mm from the surface of the brain. Each mouse was microinjected with 5 μL of autologous whole blood that was taken from the tail vein with a 1 mL syringe, over 5 minutes using a microinfusion pump (PDH 2000, Harvard Apparatus). To avoid the effect of heparin, we neither added heparin to the blood nor using a microinfusion pump (PDH 2000, Harvard Apparatus). To prevent blood clotting.

Measurement of Physiological Variables
The left femoral artery was catheterized for continuous blood monitoring (DigiMed blood pressure analyzer, MicroMed) and blood sampling for pH, blood gases (Pao2, Paco2) and glucose (Ciba Corning Diagnostics Corp).

Neurological Testing
Neurological deficits were evaluated by an observer (G.-S.C.) blinded to the age and treatment of each mouse at 6 hours and 1, 2, and 3 days after blood injection. Tests included a postural reflex test, which examines upper-body posture while the animal is suspended by the tail, and a forelimb placing test, which examines sensorimotor integration in the forelimb to visual, tactile, and proprioceptive stimuli. Neurological function was graded on a scale of 0 to 12 (normal score =0, maximal score =12) as described previously.18

Hemorrhagic Injury Analysis
Mice were euthanized 1, 3, 7 and 15 days after ICH induction and their brains were dissected out, fixed overnight in 4% paraformaldehyde in 0.1 mol/L PBS, and placed in a 30% sucrose solution for cryoprotection. Serial 30-μm-thick coronal sections were taken using a freezing microtome (Leica) and stained with cresyl violet for determination of lesion size. A digitalized image of each cresyl violet-stained brain was obtained under a light microscope (Olympus BX 51, Olympus Co), and the hemorrhagic injury area was determined using a computer-assisted image analysis program (OPTIMAS 5.1, Optimas Inc).

Identification of Microglia/Macrophages
Microglia/macrophages were identified by staining with Griffonia simplicifolia isoelectric B4. After blocking endogenous peroxidase with 0.3% H2O2 in 0.1 mol/L phosphate buffer for 10 minutes, the sections were incubated for 30 minutes in 0.1 mol/L PBS containing 10% normal horse serum and then incubated overnight at 4°C with biotinylated isoelectric B4 (1:100, Vector Laboratories; Burlingame, CA) in PBS containing 0.3% triton X-100 and 2% normal horse serum. After washing with PBS, the sections were incubated with peroxidase-conjugated streptavidin (1:200, Vector) for 1 to 2 hours at room temperature. Isolectin B4-positive cells were visualized by 5-minute incubation in 0.05 mol/L Tris-HCl buffer containing 0.02% diaminobenzidine and 0.0045% hydrogen peroxide at 37°C. Finally, the sections were dehydrated, mounted in Canada balsam, and analyzed under a bright-field microscope (Olympus BX 51, Olympus Co). Isolectin B4-positive cells were counted in 5 fields immediately adjacent to the lesion using a magnification of ×200 over a microscopic field of 0.2 mm².

Immunohistochemistry
Tissue sections were incubated with 0.3% H2O2 in PBS for 30 minutes, in PBS containing 10% normal horse serum for 30 minutes, and overnight at 4°C with polyclonal antibodies against glial fibrillary acidic protein (1:1000; DAKO Corporation; Carpinteria, CA) to detect astrocytes, myeloperoxidase (1:100; DAKO) to detect neutrophils, manganese superoxide dismutase (Mn-SOD; 10 μg/mL; Upstate Biotechnology, Lake Placid, NY), or inducible nitric oxide (iNOS; 1:1000, Chemicon, Temecula, CA), each in PBS containing 0.3% triton X-100 and 1% normal horse serum. After washing with PBS, sections were incubated sequentially with biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) and peroxidase-conjugated streptavidin (Vector), each diluted 1:200 in the same solution as the primary antiserum. To establish the specificity of each primary antibody, control sections were similarly treated in the absence of primary antibodies. After visualization with 3,3-diaminobenzidine, cells were then counted in 5 fields immedi-
ately adjacent to the lesion using a magnification of \( \times 200 \) over a microscopic field of 0.2 mm\(^2\).

**Statistical Analysis**

Data are expressed as the mean ± SEM and analyzed for statistical significance by one-way ANOVA followed by Scheffe test for multiple comparison. A probability value \( P<0.05 \) was regarded as statistically significant.

**Results**

The physiological variables tested here were not different among groups (Table). All SAM mice began to exhibit neurological signs within 1 hour after ICH and were maximally impaired within 6 hours (Figure 1). However, saline-injected mice had a score of 0 (no deficit) at all times. Neurobehavioral improvement was noted beginning on the first day after ICH in all groups, except for the old (14 month-old) SAMP8 mice (Figure 1). Three days after ICH induction, neurological deficits were still evident in the old SAMP8 group, but not in the other mice.

Mice microinjected with 5 \( \mu L \) of blood developed a large hemorrhagic injury in the striatum, with focal extension into the adjacent white matter (Figure 2). On the first day after hemorrhagic insult, there was no significant difference in the size of hemorrhagic injury in the SAMP8 and SAMR1 mice. Three days after hemorrhagic insult, however, a larger hemorrhagic injury was obtained in old SAMP8 mice (Figure 2A and 2B). Seven days after ICH induction, hemorrhagic injury was still present in old SAMP8 mice, but was present to a much lesser degree in young SAMP8 mice and young or old SAMR1 mice (Figure 2B). Similarly, the extravasation of Evans blue was not different in all experimental control groups (data not shown). However, the extent of Evans blue extravasation was markedly increased 3 days after ICH in aged SAMP8 mice, compared with other experimental ICH groups (data not shown).

The number of isolecitin B4-positive cells (ie, macrophages and microglia) was gradually increased at the ipsilateral brain of all groups after intrastrial injection of blood, beginning 1 day after injury.
The isolectin B4 immunopositive cells, which were present around or inside the hemorrhagic injury and around the needle tract in the striatum (Figure 3A) and cortex (data not shown), were round-shaped or reactive-formed. The number of isolectin B4-positive cells in older SAMP8 group was higher than the other experimental groups up to 7 days after ICH (Figure 3B). In contrast, myeloperoxidase-immunoreactive neutrophils were distributed around and inside the hemorrhagic injury in both SAMP8 and SAMR1 mice but with the numbers of cells not much different among the experimental groups (data not shown). Also, reactive astrocytes with intensified glial fibrillary acidic protein-stained processes and swollen cytoplasm did not differ in number or distribution between SAMP8 and SAMR1 mice (data not shown).

When we assayed expression of Mn-SOD, a major antioxidant enzyme, we found that, 3 days after blood injection, Mn-SOD-immunoreactive cells were distributed around the hemorrhagic injury in both SAMP8 and SAMR1 mice but with the numbers of cells not much different among the experimental groups (data not shown). Also, reactive astrocytes with intensified glial fibrillary acidic protein-stained processes and swollen cytoplasm did not differ in number or distribution between SAMP8 and SAMR1 mice (data not shown).

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Three days after blood injection, a larger number of iNOS-positive cells was observed around the site of hemorrhagic injury in older SAMP8 mice than in younger SAMP8 or younger or older SAMR mice (Figure 5). When we stained these sections with antibodies against isolectin B4 and iNOS, we found that both antibodies reacted with the same cells, indicating that the iNOS-immunopositive cells are identical to monocytes and microglia (data not shown).

**Discussion**

We have shown here that intrastriatal injection of autologous blood caused greater neurological deficit and more severe brain injury in aged SAMP8 mice, compared with younger SAMP8 mice and both younger and aged SAMR1 mice. In all experimental groups, injection of autologous blood produced tissue injury on the third day, but not on the first day. Thus, the tissue injury may be attributable to the secondary damage of the cerebral blood vessels. On the third day after blood injection, however, the size of the hemorrhagic injury in aged SAMP8 mice was double that of the other groups. The greater hemorrhagic injury in the SAMP8 old mice may reflect a greater sensitivity to injected autologous blood, not the greater hematoma. Activated monocytes/microglia expressing high levels of iNOS were abundant around and inside the brain lesions only of older SAMP8 mice. We also found that, regardless of age, the level of SOD was much less in SAMP8 mice than in SAMR1 mice. These findings suggest that the increased susceptibility of aged SAMP8 mice to the oxidative stress evoked by hemorrhagic insult may be attributable to the low capacity of their antioxidant system.

Although ICH was previously reported to cause greater neurological deficits in aged rats, we found no noticeable...
differences between young and aged SAMR1 mice. The differences in hemorrhagic injury after ICH in mice and rats may be in part a result of differences between mouse and rat microglia. For example, microglia derived from rats and mice have been shown to respond differently to thrombin, in that thrombin activated mouse microglia via a protease-activated receptor-1–dependent pathway,16 but it activated rat microglia via a protease-activated receptor-1–independent pathway.17 Our preliminary experiments also indicated that microglia derived from rats and mice showed different profiles of NO production in response to various cytokines (data not shown).

Activation of macrophages and microglia is recognized as a brain injury marker in various central nervous system diseases and may be involved in neuronal death in ischemic penumbra.18 In humans, microglia become progressively more activated with age.19 We have shown here that the number of macrophages/microglia gathering around and in-

Figure 4. ICH-induced Mn-SOD immunoreactivity. A, Mn-SOD immunoreactive cells were distributed around hemorrhagic injuries in both SAMP8 and SAMR1 mice. Mn-SOD positive cells with multiple processes were observed in striatum and cerebral cortex, but the intensity of Mn-SOD immunoreactivity was lower in SAMP8 than SAMR1 mice. Microphotographs are representative of 6 independent experiments. B, Temporal profile of the numbers of Mn-SOD positive cells near the lesions. Values are mean±SEM; n=5. ***P<0.001 compared with age-matched SAMR1 mice. Scale bar=50 μm.

Figure 5. ICH-induced iNOS immunoreactivity. A, The density of iNOS-immunoreactive cells was determined as described under the Materials and Methods. The iNOS-positive cells with round- or oval-shaped were observed around and inside the lesions, particularly in aged SAMP8 mice. Microphotographs are representative of 5 independent experiments. Scale bars=50 μm. B, Temporal profile of the number of iNOS-positive cells around the lesions. Values are mean±SEM of n=5. *** P<0.001, compared with age-matched SAMR1 mice.
side the hemorrhagic lesion after ICH was much greater in aged SAMP8 mice than in younger SAMP8 or younger or aged SAMR mice, suggesting that macrophages/microglia play a role in hemorrhagic injury. However, further experiments are needed to determine whether the effects of age on ICH-induced macrophages/microglia activation are the cause or result of the enhanced injury. Reactive macrophages/microglia were reported to mediate delayed neuronal damage after ICH and ischemia, and inhibition of their activation protected against stroke. Thus, activated macrophages/microglia may be closely associated with the increased injury after ICH in aged SAMP8 mice.

Inhibition of inflammatory substances has been reported to reduce brain injury and improve neurological deficits occurring after ICH. Recently, the productivity of inflammatory mediators, such as tumor necrosis factor-α, interleukin (IL)-1β and IL-10, has been shown to increase with age, and the protein levels of tumor necrosis factor-α, IL-1β and IL-6 in the hippocampus and cerebral cortex were markedly increased in 10-month-old SAMP mice. The aggravated hemorrhagic injury we observed after ICH in aged SAMP8 mice may be in part attributable to the increased expression of cytotoxic mediators produced by activated macrophages/monocytes.

Oxidative brain injury has been reported in both ischemic and hemorrhagic stroke. Oxidative stress in the cerebral cortex was transiently increased in aged SAMP8 mice in comparison with age-matched SAMR1 mice, and increasing oxidative stress reduced complex III activity in the respiratory chain in the SAMP8 brain. A major antioxidant enzyme, SOD, has been shown to modulate such oxidative injury, and modification of brain SOD levels has been found to affect neurological injuries after ischemic and hemorrhagic stroke. In the present study, we found that the immuno-reactivity of Mn-SOD was ~50% lower in SAMP8 mice compared with age-matched SAMR1 mice. We found, however, that the expression of SOD did not differ between younger and aged SAMP8 mice, suggesting that an additional factor(s) may be involved in augmented injury in aged SAMP8 mice.

Further, we found that the number of iNOS-positive cells was much greater in aged SAMP8 mice than in young SAMP8 or young or aged SAMR mice, and that these iNOS-expressing cells were identical to activated monocytes/microglia. We previously reported that interferon-γ, plus IL-1β or lipopolysaccharide, synergistically increased ischemia/glucose deprivation-induced neuronal cell injury through the over-expression of iNOS. Increased expression of iNOS and glucose deprivation rapidly depleted intracellular reduced glutathione, resulting in an accumulation of a strong cytotoxic mediator, ONOO−, inside the cells. The level of reduced glutathione has also been reported to be decreased during early stages of aging, resulting in acceleration of the dysfunction of mitochondrial electron transport and neurodegeneration. In addition to the lower SOD activity, therefore, the increased level of iNOS may be a main cause of increased hemorrhagic injury in aged SAMP8 mice.

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