Characterization of the Microglial Response to Cerebral Ischemia in the Stroke-Prone Spontaneously Hypertensive Rat

Louise Marks, Hilary V.O. Carswell, Elaine E. Peters, David I. Graham, James Patterson, Anna F. Dominiczak, I. Mhairi Macrae

Abstract—Stroke-prone spontaneously hypertensive rats (SHRSP) sustain more ischemic damage after middle cerebral artery occlusion than do their reference strain, the Wistar-Kyoto rat (WKY). The cause of increased stroke sensitivity is still under investigation. In general, SHRSP display a greater response to inflammatory stimuli than do WKY. Because inflammatory cells may influence the extent of damage in experimental stroke, this study has investigated the acute inflammatory response to focal ischemia in SHRSP and WKY. Adult male SHRSP (n=5) and WKY (n=5) were anesthetized and underwent distal middle cerebral artery occlusion. After 24 hours of recovery, infarct volume, neutrophil counts, and activated microglia counts were performed. SHRSP displayed more ischemic damage than did WKY (135±4.7 versus 102±4.7 mm³ [mean±SEM], P<0.005). Brain neutrophil counts were extremely low in both strains. SHRSP displayed significantly more activated microglia than did WKY in the ipsilateral hemisphere (respective SHRSP versus WKY values [mean±SEM] were 88±3.6 versus 51±3.4 per mm² for the cortical peri-infarct region [P<0.005] and 183±7.9 versus 156±3.7 per mm² for the infarct core [P<0.05]) and in the contralateral hemisphere (eg, respective SHRSP versus WKY values were 102±3.2 versus 50±3.1 per mm² for the sensorimotor cortex [P<0.0001]). No neutrophils and very few activated microglia were found within the brains of naive rats. However naive SHRSP possessed more microglia (resting and activated) than did naive WKY. This study demonstrates a more pronounced microglial response to focal ischemia in SHRSP compared with WKY and provides evidence of a potential role for inflammatory processes in response to ischemic damage. (Hypertension. 2001;38:116-122.)

Key Words: rats, inbred SHRSP ■ ischemia ■ stroke ■ occlusion ■ genetics

Stroke-prone spontaneously hypertensive rats (SHRSP) have a genetically determined increased sensitivity to experimental stroke and display greater volumes of cerebral infarction than do their normotensive reference strain, Wistar Kyoto rats (WKY).¹

Proposed contributory factors for increased stroke sensitivity in SHRSP include impaired collateral blood supply through cerebrovascular anastomoses,² increased glutamate release,³ and genetic hypertension.⁴ However, because inflammatory cells have been proposed to contribute to cerebral ischemic damage⁵ and because SHRSP, compared with WKY, have a more pronounced inflammatory response in the periphery, an elevated inflammatory response to cerebral ischemia may represent an additional contributory factor.

In response to a provocative dose of lipopolysaccharide, SHRSP and spontaneously hypertensive rats (SHR, the strain from which the SHRSP were derived) both produce significantly more tumor necrosis factor (TNF), a mediator of inflammation, than do WKY.⁶ Compared with WKY, mature SHR have significantly elevated neutrophil, monocyte, and lymphocyte counts in their blood,⁷ with an abnormal degree of activation of these cells, and they produce higher levels of TNF and platelet-activating factor in cerebrospinal fluid after intravenous lipopolysaccharide administration.⁸ Neutrophils have been proposed to contribute to ischemic injury, migrating to the site via a process of rolling and tethering to the blood vessel wall through the expression of adhesion molecules on their surface and on the endothelium of blood vessels.⁹ Once adhered, neutrophils can cause damage not only by plugging blood vessels and reducing blood flow but also by their release of cytotoxic substances at the endothelial surface or after migration into the brain.

Microglia, resident within the brain and constituting 5% to 20% of the total glial cell population,¹⁰ also contribute to the inflammatory response to ischemia. Their primary purpose as phagocytic cells is to remove debris after irreversible damage, and their activation has also been proposed to be both detrimental and beneficial in certain disorders of neurodegen-

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eration, inflammation, and ischemia. In the normal central nervous system (CNS), microglia are present in a resting state and possess a characteristic ramified morphology. With the onset of CNS injury, microglia become activated, and they proliferate and migrate toward the site of damage. Activation is associated with a change to a more proinflammatory profile.14 and once activated, microglia are capable of secreting profibrotic substances such as glutamate, nitric oxide (NO), reactive oxygen intermediates (O2− and H2O2), matrix metalloproteinases and their inhibitors, hydrodases, cathepsins, plasminogen activators, and cytokines (interleukin-1, interleukin-6, and TNF-α).14 It is the release of cytokotins that is proposed to be the main cause of microglia-and neutrophil-mediated damage during and after ischemia. Antibodies directed against TNF-α and interleukins, for example, have been shown to significantly attenuate ischemic damage.

In addition to the proposal that inflammatory cells exacerbate damage, there is also evidence of a beneficial role involving certain inflammatory cells in promoting repair and recovery after peripheral or CNS injury. Phagocytosis of cellular debris from degenerated neurons supports wound healing and neuronal regrowth, and secretion of certain cytokines and growth factors can aid neuronal regeneration. Therefore, the aims of the present study were to use quantitative methods to characterize the CNS inflammatory response to focal ischemia in SHRSP and WKY and to determine whether strain differences exist in the number of neutrophils and activated microglia present in naive animals and after experimental ischemia.

**Methods**

All experiments were carried out with a license from the Home Office and were subject to the Animals (Scientific Procedures) Act, 1986. Age-matched (3- to 5-month) male rats were obtained from inbred colonies of SHRSP and WKY that were held in the Department of Medicine and Therapeutics, University of Glasgow.19

**Induction of Focal Cerebral Ischemia**

Anesthesia was induced with 5% halothane in an oxygen–nitrous oxide mix (30:70) and maintained at 1% to 1.5%. Blood gases, plasma glucose, and blood pressure were monitored throughout the procedure. Body temperature during surgery was monitored via a rectal probe, and brain temperature was measured by using a needle probe inserted into the temporalis muscle. A distal occlusion of the middle cerebral artery (MCA) was carried out via a transorbital approach by using a modified version of the method of Tamura et al.20 (1981). The dura was removed, and a 1.5- to 2-mm segment of artery was electrocoagulated and cut just distal to the inferior cerebral vein. The wound was sutured, anesthesia was withdrawn, and the animal was allowed to recover for 24 hours. Ischemic and naive control animals were deeply anesthetized before transcardiac perfusion with 4% paraformaldehyde in PBS. The skull was then placed in 4% paraformaldehyde for 24 hours, after which time the brain was removed from the skull and placed into 30% sucrose solution for 2 to 3 days for cryoprotection. Brains were then frozen at −42°C in isopentane for 5 minutes, and 30-μm coronal cryostat sections were collected on poly-l-lysine–coated slides. Sections were cut throughout the MCA territory for infarct determination and immunohistochemistry.

**Infarct Determination**

Sections taken at 8 coronal levels throughout the MCA territory were stained with hematoxylin and eosin, and the infarct was transcribed onto line diagrams. The infarct area was measured by using image analysis (MCID, Imaging Research), and infarct volume was calculated by integration.

**Neutrophil Counts**

Neutrophils, identified by their morphology and the characteristic segmented or lobular appearance of their nuclei, were counted on hematoxylin-eosin–stained sections at the level of the nucleus accumbens, globus pallidus, and lateral habenula.

**Immunohistochemistry**

Sections at 8 preselected coronal levels were stained with the monoclonal microglial antibody Ox-42 (1:1000, Serotec) or the polyclonal antibody mrf-1 (1:100, a gift from Drs Tanaka and Koike, Molecular Neurobiology Laboratory, Hokkaido University, Hokkaido, Japan). To ensure consistency of staining, SHRSP and WKY sections were always stained in the same immunohistochemical run, with appropriate negative and positive control sections. Negative controls involved the omission of the primary antibody. Sections from a rat brain exhibiting signs of meningitis (from a separate series of experiments) were included as positive inflammatory control sections. Sections were washed twice for 5 minutes each with 50 mmol/L PBS (Oxoid), treated with 0.2% Triton X-100 (Sigma Chemical Co) for 30 minutes, and then treated with 0.3% H2O2 in methanol for 20 minutes to quench endogenous peroxidase activity. Blocking sera, 10% normal horse serum for Ox-42 or 10% normal goat serum for mrf-1 (Vector Laboratories), were applied for 1 hour before an overnight incubation with the primary antibody (1:1000, Ox-42; 1:100, mrf-1). The secondary antibody (rat-adsorbed anti-mouse IgG raised in horse, 1:100 for Ox-42, or rat-adsorbed anti-rabbit raised in goat, 1:100 for mrf-1) was then applied for 1 hour. Sections were treated with a Vectastain ABC kit for 1 hour and a DAB peroxidase substrate kit (Vector Laboratories) and counterstained with hematoxylin.

**Quantification of Inflammatory Cell Staining**

Activated microglia, as determined by positive staining, the presence of a nucleus, and appropriate morphology, were counted in 3 nonoverlapping regions of 0.25 mm2 in the following brain regions: cortical infarct core, parietal cortex (peri-infarct), cingulate cortex, genu of the corpus callosum, and the external capsule in the ipsilateral hemisphere and homotopic regions of the contralateral hemisphere. Values were expressed as activated microglia/mm2. Staining distribution maps were produced by marking areas of activated microglial staining onto line diagrams displaying the 8 preselected levels. The 5 maps for each strain were superimposed to give an average activated microglial distribution with use of the NIH

**Physiological Variables Monitored During Distal MCAO in WKY and SHRSP**

<table>
<thead>
<tr>
<th>Rat Strain</th>
<th>Weight, g</th>
<th>MABP, mm Hg</th>
<th>PaCO2, mm Hg</th>
<th>pH</th>
<th>Body Temperature, °C</th>
<th>Brain Temperature, °C</th>
<th>Plasma Glucose, mmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>WKY MCAO (n=5)</td>
<td>336±14</td>
<td>87±3</td>
<td>40.0±1.7</td>
<td>7.40±0.02</td>
<td>36.85±0.44</td>
<td>36.85±0.56</td>
<td>10.2±0.5</td>
</tr>
<tr>
<td>SHRSP MCAO (n=5)</td>
<td>262±11†</td>
<td>123±9†</td>
<td>37.0±1.6*</td>
<td>7.45±0.01*</td>
<td>37.24±0.14</td>
<td>36.2±0.14</td>
<td>9.6±0.8</td>
</tr>
</tbody>
</table>

Values are mean±SEM and were analyzed by unpaired 2-tailed Student’s t test. MABP indicates mean arterial blood pressure; MCAO, MCA occlusion.

*P<0.05, †P<0.01.
image program. Similar diagrams were prepared for the distribution of the infarct for comparison with the distribution of activated microglia.

Statistical Analysis
Data are presented as mean±SEM. The significance of differences in microglial number, infarct size, and physiological variables between strains was determined by unpaired Student’s t test.

Results
Physiological Parameters
Physiological parameters were maintained within normal limits under anesthesia (Table): normocapnia (36 to 42 mm Hg), physiological pH (7.4), and normal body temperature (36.5°C to 37.5°C). Plasma glucose levels and brain and body temperatures were similar in the 2 strains, but as expected, SHRSP exhibited lower body weight and significantly higher mean arterial blood pressure under anesthesia than did WKY. Differences in PaCO₂ and pH were statistically but not physiologically significant.

Infarct Size
Compared with WKY, SHRSP displayed a greater degree of ischemic damage (Figure 1). Infarct volume was 135±4.7 mm³ in SHRSP compared with 102±4.7 mm³ in WKY (P<0.005, unpaired Student t test). The distribution and extent of ischemic damage in each strain are illustrated on the left side of Figure 2.

Neutrophil Counts
No neutrophils were observed within blood vessels of the MCA territory in either SHRSP or WKY after MCA occlusion. No neutrophils were identified within the parenchyma of WKY, and only one of the 5 SHRSP displayed neutrophils within MCA territory. In this animal, 3 neutrophils were identified in the cortex at the level of the nucleus accumbens, 2 were identified at the level of the globus pallidus, and 4 were identified at the level of the lateral habenula.

Microglia
Activated microglia, as determined by immunopositive staining and by morphology under the light microscope, were present in both SHRSP and WKY after MCA occlusion. Activated microglia had short projections and more distinct, densely stained cell bodies compared with the long spindly projections and faintly stained cell bodies of resting microglia (Figure 3). Distribution of the activated microglia included brain regions in the ipsilateral and the contralateral hemispheres in both gray and white matter. As previously reported, the morphology of the microglia differed between gray and white matter, with the gray matter microglia possessing a stellate morphology and microglia in the white matter appearing more bipolar.

Consequence of Ischemia
Activated microglia were evident in both ipsilateral and contralateral hemispheres of both strains after 24 hours of focal cerebral ischemia, with the greatest density of cells being within the infarct core (Figure 2 and Figure 4, left.)

![Figure 1](image1.png)
Figure 1. Volume of ischemic damage in SHRSP (n=5) and WKY (n=5) at 24 hours after MCA occlusion. Data represent volume of ischemic damage, analyzed by 2-tailed unpaired Student’s t test. **P<0.005.

![Figure 2](image2.png)
Figure 2. Composite line diagrams of the topography of the infarct and activated microglial densities for WKY (n=5) and SHRSP (n=5). In the left diagram (scale 0 to 4), 4 represents areas that were infarcted in 100% of animals; 3, areas infarcted in 75% of animals; 2, areas infarcted in 50% of animals; and 1, areas infarcted in 25% of animals. In the right diagram, the scale 0 to 12 represents activated microglial cell densities, with 12 representing the greatest density of cells.
Staining intensity was, in general, stronger in the ipsilateral hemisphere. The microglia (activated and resting) did not differ in morphology between the 2 hemispheres; differences were found only in their frequency and intensity of staining.

Microglial Counts: Strain Differences
SHRSP exhibited greater numbers of activated microglia than did WKY in all 5 brain regions examined in both hemispheres (Figure 4). In the ipsilateral hemisphere, the strain difference was significant in the histologically normal peri-infarct region of the cortex surrounding the infarct, in the adjoining region of the cingulate cortex (also histologically normal and supplied by the anterior cerebral artery), and within the infarct core itself (Figure 4, left). Although activated microglial counts overall were lower in the contralateral hemisphere, a strain difference between SHRSP and WKY was clearly evident (Figure 4, right). The composite line diagrams (Figure 2) display the greater density of cells and their more widespread distribution in SHRSP compared with WKY. Staining intensity was also greater within the ipsilateral hemisphere and more specifically within the coronal levels nearest to the core of MCA territory. The diagrams also show regional microglial distribution within gray and white matter.

Naive Controls
Activated microglia were extremely rare in the MCA territory of naive control rats of either strain (Figure 5, right). However, compared with WKY brains, naive SHRSP brains had higher total numbers of microglia (resting and activated) in all of the brain regions examined over the 8 coronal levels (Figure 5, left). This difference in microglial number between SHRSP and WKY was statistically significant in the 2 white matter regions examined, the external capsule and the genu of the corpus callosum.

Figure 4. Activated microglial counts 24 hours after MCA occlusion in ipsilateral (left) and contralateral (right) hemispheres. Data are represented as mean±SEM (n=5 per group) and were analyzed by 2-tailed unpaired Student’s t test. *P<0.05, **P<0.005, and ***P<0.001.

Figure 3. a, Activated microglia in gray matter. b, Resting microglia in gray matter. c, Activated microglia in white matter. d, Resting microglia in white matter. Bar=50 μm.
Discussion

Quantitative analysis of the inflammatory response to focal cerebral ischemia in the genetically determined, stroke-sensitive SHRSP rat has revealed 2 important results: (1) SHRSP displayed significant increases in the numbers of activated microglia associated with the site of injury compared with the WKY reference strain 24 hours after ischemia, and (2) there was no evidence of a significant neutrophil involvement in the evolution of the infarct in either strain at this time point. Compared with WKY brains, control brains from SHRSP also displayed a greater density of microglia, especially in white matter tracts. These results are important in terms of improving our understanding of genetically determined “stroke sensitivity” and of the role of inflammatory cells in stroke, which are research areas associated with many unresolved questions and opposing views.

Stroke-sensitive rat strains such as the SHR and SHRSP have previously been shown to display an elevated inflammatory response to inflammatory stimuli, suggesting that an amplified response to cerebral ischemia may also be associated with increased stroke sensitivity. We tested this hypothesis in the present study and found no significant neutrophil presence in cerebral vessels or parenchyma of either SHRSP or WKY at the 24-hour time point. Previous studies from this laboratory have similarly failed to find evidence of neutrophils in acute focal ischemic damage. However, widespread microglial activation was evident within 24 hours of focal ischemia in both strains (Figure 2), in agreement with previous studies of global and focal ischemia in standard rat strains.

SHRSP exhibited greater microglial activation than did WKY in terms of the density and distribution of cells (Figures 3 and 4), supporting a potential link with increased stroke sensitivity in the SHRSP. What is not yet clear, however, is whether the greater microglial activation is caused by or contributes to the increased infarct size in the SHRSP. There was no significant correlation between infarct size and activated microglial density in either the infarct core or the peri-infarct cortex ($r^2=0.158$, $P=0.507$ for infarct core; $r^2=0.324$, $P=0.405$ for peri-infarct cortex). This indicates that (1) the greater microglial activation is probably not a direct result of the increased infarct size, and (2) the lack of a correlation does not rule out a contributory role of activated microglia to increase infarct size, but if they are involved, they may be one of a number of contributors.

At 24 hours, the infarct is still evolving in the SHRSP, and tissue in the peri-infarct region is balancing between life and death. Although it is widely reported that the inflammatory response may contribute to brain damage, potentiating the destructive effects of ischemia, microglia could alternatively have a beneficial influence, representing the brain’s attempt to protect itself from further damage, as happens in the periphery (discussed below).

Activated microglia could contribute to ischemic damage both directly (via synaptic stripping and neurophagia) and indirectly (through the release of cytotoxins). Cytotoxins induce lipid peroxidation, excess release of transmitters and hormones, vascular leakage, edema, necrosis, and changes in ion flow. Glutamate released from microglia can give rise to excessive N-methyl-D-aspartate receptor activity, resulting in neuronal cell death. High extracellular potassium levels, as found in ischemic tissue, potentiate glutamate release from microglia that are particularly sensitive to changes in potassium levels because of their lack of a substantial potassium outward current.

A recent study has suggested that microglia are capable of contributing to the development of the ischemic infarct at time points similar to those in the present study. However, the conclusion was based on the expression of interleukin-1β and Bax in microglia within the zone of injury rather than from the results of an intervention strategy. The definitive experiment, to determine whether microglia contribute to or ameliorate ischemic injury, would be to selectively block microglial activation without affecting the other deleterious mechanisms in the ischemic cascade. However, although theoretically feasible, this is not currently achievable because potential drugs and strategies that attenuate microglial activation have significant influences elsewhere in the ischemic cascade. For example, tetracycline derivatives, which block microglial activation, also display antagonism of glutamate, cyclooxygenase-2, interleukin 1–converting enzyme, inducible NO synthase, gelatinase B, and superoxide production by leukocytes.

After neuronal injury in the periphery, macrophages participate in neuronal regeneration by performing phagocytosis of debris to promote neuronal regrowth. Activated...
microglia, like macrophages, are also recruited into the injured brain to remove debris. In the present study, the highest microglial counts were found in the ipsilateral hemisphere within the infarct in both strains, which would support a phagocytic role.

Microglia also secrete growth factors that promote the migration of astrocytes, which are glia that are conducive to neuronal regrowth and wound healing. In addition to stimulating cell migration and/or proliferation, these growth factors may also increase protease activity or alter the expression of extracellular matrix components.

The participation of microglia in immune activities and regeneration of neurons within the CNS, however, is much less intense than that seen for macrophages in cases of peripheral injury. This is possibly because of the presence of inhibitory substances that restrict their participation and produce an inhospitable growth environment within the CNS. In spite of a less pronounced phagocytic response in the CNS, microglia have been reported to perform a beneficial role in CNS injury after viral infection and axonal injury.

Activation and migration of microglia may be induced by chemokines or cytotoxins released during ischemia, which would explain the observed increase in numbers of activated microglia in the ipsilateral hemisphere of both strains. A rise in cytokine levels, occurring 1 to 2 days after ischemia, correlates well with the time scale for microglial activation. Significant numbers of activated microglia were also recorded in more distant sites in the contralateral hemisphere in the present study and others. Explanations for contralateral activation of microglia include active recruitment by signals sent from the ipsilateral microglia. A significant increase in the number of activated microglia in the contralateral genu of the corpus callosum may be representative of migrating microglia moving along the white matter tracts to reach their target site, which is, in this case, the area of ischemic damage. Cytokines secreted from the ipsilateral hemisphere may stimulate activation, proliferation, and migration of contralateral microglia to the site of damage. Alternatively, contralateral microglia may become activated as a result of diaschisis or as a result of waves of ischemia-induced spreading depression. Because spreading depression activates astrocytes and microglia and because SHRSP exhibit a greater amount of spreading depression than do WKY after ischemia, this phenomenon provides a possible explanation for the greater number of activated microglia in the contralateral hemisphere of SHRSP compared with WKY. Increases in brain swelling, significant in this model at 24 hours, represent an alternative stimulus for contralateral activation of inflammatory cells and mediators.

Strain differences in the number of activated microglia after ischemia could be a consequence of differences in basal levels of resting or activated microglia. SHRSP are known to develop hypertensive blood pressure within weeks of birth, resulting in a systolic blood pressure of \( \approx 200 \text{ mm Hg} \), which is maintained throughout life. It is possible that this underlying hypertension could influence the levels of activated microglia under basal conditions. Indeed, hypertension in SHRSP has been shown to predispose the strain to blood-brain barrier breakdown. Blood-brain barrier breakdown could provide changes in the microenvironment that could stimulate microglial activation under nonischemic conditions with no equivalent activation occurring in the normotensive WKY. The present study showed that the numbers of activated microglia within the MCA territory of naive SHRSP and WKY were extremely small, with no significant elevation in SHRSP. However, microglial counts per se (resting and activated combined) were elevated in naive SHRSP, with significantly greater numbers in the 2 white matter regions studied. Therefore, the presence of a greater number of basal resting microglia could be a contributory factor in the greater numbers of activated microglia seen in SHRSP after ischemia.

To conclude, the present study has shown evidence of an elevated microglial response to experimental ischemia in SHRSP compared with WKY, with greater numbers of resting microglia in SHRSP under basal conditions. These results illustrate an increased inflammatory response to focal cerebral ischemia in SHRSP and suggest a role for increased microglial activation in stroke sensitivity. What remains unclear at present is whether these microglia have a beneficial or detrimental role within the evolving infarct.

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