Hypoxic-Ischemic Injury Induces Macrophage Inflammatory Protein-1α Expression in Immature Rat Brain

Rita M. Cowell, BS; Haiyan Xu, MS; John M. Galasso, PhD; Faye S. Silverstein, MD

Background and Purpose—Macrophage inflammatory protein (MIP)-1α is a well-characterized monocyte chemoattractant; its role in regulating monocyte and microglial recruitment and activation in the injured neonatal brain is unknown. We evaluated the impact of acute hypoxic-ischemic (HI) brain injury on the expression of MIP-1α in neonatal rat brain.

Methods—To elicit forebrain ischemic injury, 7-day-old (P7) rats underwent right carotid ligation, followed by 2.5 hours of 8% oxygen exposure. We used an enzyme-linked immunosorbent assay and immunochemistry to detect MIP-1α; double-labeling immunofluorescence assays were analyzed with confocal microscopy to identify cellular sources of MIP-1α. Immunocytochemistry assays were also used to detect 2 MIP-1α receptors, CCR1 and CCR5.

Results—We found marked increases in tissue concentrations of MIP-1α in the HI cerebral hemisphere, peaking from 8 to 72 hours after lesioning. Immunocytochemistry assays revealed that MIP-1α was constitutively expressed in physiologically activated microglia; from 8 to 120 hours after lesioning, MIP-1α immunoreactive monocytes and microglia accumulated in the lesion territory. In immunoreactive cells, MIP-1α was diffusely distributed throughout the cytoplasm at early post-HI time intervals; by 72 hours, MIP-1α immunoreactivity was typically concentrated adjacent to the nucleus, a pattern indicative of active MIP-1α production. In P7 to P12 brain, many cells expressed MIP-1α receptors; both CCR1 and CCR5 immunoreactivity were localized to endothelium and ependyma; CCR1-immunoreactive astrocytes and neurons and CCR5-immunoreactive microglia were also identified.

Conclusions—These data implicate MIP-1α as a mediator of the complex and sustained inflammatory response initiated by perinatal HI brain injury. (Stroke. 2002;33:795-801.)

Key Words: astrocytes ■ chemokines ■ endothelium ■ neonate ■ receptors, chemokine ■ rats

Diverse inflammatory mediators contribute to the pathophysiology of cerebral ischemia/reperfusion injury. A number of anti-inflammatory interventions are neuroprotective in animal models of ischemia, ranging from agents with clearly defined modes of action such as interleukin 1 receptor antagonist to drugs with imprecisely defined anti-inflammatory properties, such as minocycline.

Recent epidemiological data stimulated interest in the roles of inflammation in neonatal brain injury. In animal models of neonatal stroke, hypoxic-ischemic injury elicits an acute inflammatory response, characterized by increased expression of proinflammatory mediators, a rapid microglial/microcytic response and gliosis; inflammation persists for at least 5 weeks.

β-Chemokines, including macrophage inflammatory protein-1α (MIP-1α) and monocyte chemoattractant protein-1 (MCP-1), have been implicated as potential modulators of the acute inflammatory response in the brain. Initial evidence that MIP-1α plays a role in brain pathology was provided by results of a study in which treatment with MIP-1α antibody improved the outcome of experimental autoimmune encephalomyelitis. In experimental models of cerebral ischemia, several studies have reported increased MIP-1α mRNA expression, both in mature and neonatal rats, and 1 study documented MIP-1α protein 4 hours after a transient focal ischemic insult in the adult rat.

In neonatal rodents, hypoxic-ischemic brain injury results in MCP-1 expression by multiple cell types in the lesion territory. In contrast with results reported in adult stroke models, in which astrocytes were the predominant source of MCP-1, in neonates, injured neurons were a major source of MCP-1. This finding suggested that maturational stage could influence the chemokine response to acute brain injury and prompted us to evaluate the impact of an acute hypoxic-ischemic insult on MIP-1α expression in neonatal rodent brain.

Two MIP-1α receptors, CCR5 and CCR1, have been identified in brain or in brain-derived cells. CCR5 is expressed by human fetal astrocytes and neurons, microglia, and cerebral endothelium. CCR1 has been identified on...
microglia, on astrocytes, and in cerebral microvessels. To identify potential targets of MIP-1α in the injured neonatal brain, we also evaluated the cellular distributions of CCR1 and CCR5 at this developmental stage.

Materials and Methods

Materials

Reagents used for enzyme-linked immunosorbent assay (ELISA) included affinity-purified anti-MIP-1α Ab (PeproTech), recombinant MIP-1α (Biosource), and horseradish peroxidase-conjugated neutraltide–avidin (Southern Biotechnology Associates). Primary antibodies and reagents for immunohistochemistry included rabbit anti-rat MIP-1α (1:1000; Peprotex), goat anti-rat CCR5 (1:400; Santa Cruz Biotechnology), goat anti-rat CCR1 (1:500; Santa Cruz Biotechnology), mouse anti-NeuN (1:1000; Chemicon), fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit IgG (1:200 to 1:500; Vector Laboratories), and donkey anti-sheep IgG (1:200; Jackson Immunoresearch). All other materials were from Sigma.

Materials and Methods

Surgery

In 7-day-old (P7) methoxyfluorane-anesthetized Sprague-Dawley rats, right carotid artery was isolated, doubly ligated, and transected. After recovery in an incubator (37 °C), animals were placed in warmed chambers (36 °C) and exposed to 8% O2/92% N2 for 1 to 2.5 hours. Animals were returned to their dams when they resumed normal activity (within 30 minutes). Procedures were approved by the University of Michigan Committee on the Use and Care of Animals in Research; all efforts were made to minimize animal suffering and the number of animals used.

Enzyme-Linked Immunosorbent Assay

Animals were killed by decapitation; the cerebral hemispheres were separated on ice. Each hemisphere was homogenized in 500 µL PBS and centrifuged (13,000 rpm, 25 minutes, 4 °C); supernatants were stored at −70 °C. Supernatant protein content was assayed (BCA kit; Pierce).

To evaluate the peak and duration of MIP-1α expression, samples were collected at 0 to 120 hours after hypoxia-ischemia (0 hour, n = 4; 4 hours, n = 4; 8 hours, n = 4; 16 hours, n = 8; 72 hours, n = 4; 120 hours, n = 4). To evaluate the relationship between the duration of the hypoxic-ischemic insult and the magnitude of stimulation of MIP-1α, samples were also obtained at 16 hours from animals that had developed to measure MCP-1. Microtiter plates (96-well; 1 hour, 2.5 hours, 8 hours, 72 hours, 120 hours) were coated with 1 µg/well of affinity-purified anti-MIP-1α antibody diluted in 0.1 mol/L Na2HPO4 (pH 9.1; 24 hours at 4 °C). Plates were washed (PBS/0.05% Tween 20), incubated in blocking buffer (PBS/0.05% Tween-20/1% BSA [200 µL/well; 2 hours, 25 °C]), and washed. Samples were loaded in duplicate (95 µL/well; 24 hours, 4 °C). After 3 washes, plates were incubated with biotinylated anti-MIP-1α antibody (0.02 µg/100 µL/well; 1 hour, 25 °C), washed, and then incubated with horseradish peroxidase-conjugated neutralite–avidin (100 µL/well; 1:4000; 30 to 45 minutes, 25 °C). Chromogenic detection was performed using ABTS (Boehringer Mannheim). Optical density at 405 nm was measured using an automated plate reader; MIP-1α content was derived from a standard curve, generated using recombinant rat MIP-1α. Concentrations of recombinant MIP-1α as low as 10 pg/mL were detected; the rectilinear relation between log concentration and log OD extended up to 5 ng/mL. Values, expressed as nanogram MIP-1α/gram protein, were compared using the Kruskal-Wallis test.

Immunohistochemistry

Antibodies were diluted in PBS/5% serum/0.3% Triton X-100. Sections were washed (PBS, 5 minutes), blocked in 10% normal serum and goat anti-rat MIP-1α (1:100; Santa Cruz). Secondary antibodies included biotinylated horse anti-mouse IgG, horse anti-rat IgG, goat anti-rabbit IgG (1:200 to 1:500; Vector Laboratories), and donkey anti-sheep IgG (1:200; Jackson Immunoresearch), and FITC-labeled donkey anti-goat IgG, FITC-labeled donkey anti-rabbit IgG, FITC-streptavidin, rhodamine-labeled donkey anti-mouse IgG, rhodamine-labeled donkey anti-goat IgG, CY-5-labeled donkey anti-rabbit IgG, and CY-5-labeled donkey anti-mouse IgG (1:200; Jackson Immunoresearch). Goat and horse sera were purchased from Vector Laboratories and donkey serum from Jackson Immunoresearch. All other materials were from Sigma.

Tissue Preparation for Microscopy

Animals were deeply anesthetized with pentobarbital at 6 sequential time intervals after lesioning (8, 16, 24, 48, 72, or 120 hours; n = 4 to 6/group) and perfused with 10 mL PBS, followed by 10 mL 4% paraformaldehyde/PBS. Samples were also obtained from age-matched controls (P7, P8, P10, P12; n = 2 to 3/group) and adults (P42, n = 3). Brains were post-fixed in paraformaldehyde, cryoprotected in graded sucrose solutions, embedded (Histoprep; Fisher), frozen, and stored at −80 °C. Frozen sections, obtained at the levels of mid-striatum and dorsal hippocampus, were mounted on gelatin-coated slides (15-µm sections for light microscopy; 30-µm sections for confocal microscopy). Slides were dried overnight and either used the next day or stored at −80°C; sections, stored for up to 6 weeks, retained MIP-1α, CCR1, and CCR5 signal.

Distribution Mapping

To compare the neuroanatomical distribution of neuronal injury, MIP-1α expression, and ED-1 immunostaining, representative hippocampal sections (4/group) were selected. NeuN-stained sections were viewed (10× objective), and areas of condensed, pale, and absent neurons were mapped onto a tracing of the hippocampus. Immunoreactive cells were counted either in every 10× field (to evaluate cytoplasmic MIP-1α and ED-1) or in every 40× field (to identify juxtanuclear MIP-1α) across an entire section and represented by dots mapped at the corresponding sites.

Confocal Microscopy

In preliminary immunofluorescence assays, we found that with polyclonal antibodies an amplification step resulted in unacceptable increases in background fluorescence. Therefore, we used 10-fold higher concentrations of each primary antibody than those listed in Materials and Methods. TOTO-3, a nucleic acid stain, was used to identify nuclei.

After incubation with the primary antibody (18 hours, 4 °C), sections were washed (PBS, 30 minutes), incubated with secondary antibody (2 hours, 25 °C), washed (PBS, 30 minutes), and over-sipped with anti-fade media (Molecular Probes). To identify NeuN immunoreactive neurons, an amplification step was necessary; after incubation with anti-NeuN antibody (1:100), sections were incubated
Results

Neuronal Injury

We incorporated immunoassays using the anti-NeuN antibody to delineate the evolution of neuronal injury; in P7 to 12 rat brain, there was both nuclear and cytoplasmic neuronal immunostaining. Figure 1 compares NeuN immunostaining in normal P7 brain (Figure 1A) and in animals evaluated at 5 sequential time intervals after lesioning (Figure 1B through 1F). There was a relatively rapid disruption of NeuN immunostaining in the first 72 hours after lesioning; of note, the timing of loss of immunostaining differed among regions within the lesion territory. At 8 hours after lesioning, loss of NeuN immunoreactivity was evident in the habenula and thalamus (Figure 1B). At 16 hours, loss of immunostaining extended to the cortex, thalamus, and striatum (Figure 1C). At 24 hours, note ipsilateral hemisphere swelling. E, At 48 hours, extensive hippocampal neuronal loss is evident in the pyramidal cell layer (arrowhead) and dentate gyrus (arrow). F, At 72 hours, note hippocampal injury both in the CA1 (arrow) and CA3 (arrowhead) subfields and subtle neuronal loss in the contralateral cortex (†). Scale bar, 1 mm.

with biotinylated horse anti-mouse IgG (2 hours, 25°C) and then with FITC-streptavidin.

Immunofluorescence was visualized in dual channel mode on a Nikon Diaphot 200 microscope equipped with a Noran confocal laser scanning imaging system and Silicon Graphics Indy workstation. Images were processed using Adobe Photoshop 5.0; in some cases, the color of the label was changed to facilitate visualization of colocalization.

Each experiment included controls in which an equal concentration of species-matched IgG was substituted for the primary antibody. In preliminary experiments, we noted nonspecific staining in the cortex of severely lesioned brains evaluated at 48 to 72 hours after hypoxia-ischemia; this was minimized by increasing the serum concentrations of MIP-1α from 4 to 16 hours after lesioning (183±67 ng/g protein at 16 hours). In samples from the

MIP-1α ELISA

Tissue homogenate assays of MIP-1α (see Table) demonstrated marked changes in MIP-1α concentrations in samples obtained from the lesioned hemisphere 0 to 120 hours after hypoxia-ischemia (P<0.02, Kruskal-Wallis test). Immediately after hypoxia-ischemia, MIP-1α was barely detectable (0.3±0.0 ng/g protein); there were marked increases in tissue concentrations of MIP-1α from 4 to 16 hours after lesioning (183±67 ng/g protein at 16 hours). In samples from the

<table>
<thead>
<tr>
<th>Duration of HI (h)</th>
<th>N</th>
<th>Contralateral</th>
<th>Ipsilateral†</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>2±1</td>
<td>4±3</td>
</tr>
<tr>
<td>1.5</td>
<td>6</td>
<td>2±1</td>
<td>68±37</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>2±0</td>
<td>60±40</td>
</tr>
<tr>
<td>2.5</td>
<td>6</td>
<td>4±2</td>
<td>138±51</td>
</tr>
</tbody>
</table>

P7 rats underwent right carotid artery ligation, followed by exposure to 8% O2. Samples were prepared from the left (“contralateral”) and right (“ipsilateral”) cerebral hemispheres. Panel A data were from animals exposed to 2.5 hours of 8% O2 and killed 4 to 120 hours later. Panel B data were from animals exposed to 8% O2 for 1 to 2.5 hours, and killed 16 hours later. Tissue content of MIP-1α was measured using an enzyme-linked immunosorbent assay. Values are expressed as mean ng/g protein±SEM; intergroup differences were evaluated using Kruskal-Wallis tests.

†P<0.05 comparing contralateral hemisphere values.

‡P<0.02, comparing ipsilateral hemisphere values.

§P<0.01, comparing ipsilateral hemisphere values.

Hypoxia-Ischemia (HI) Stimulates MIP-1α Production in Neonatal Rat Brain

A. Time course of HI-induced MIP-1α production

<table>
<thead>
<tr>
<th>Time after HI (h)</th>
<th>N</th>
<th>Contralateral*</th>
<th>Ipsilateral†</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2</td>
<td>0±0</td>
<td>0±0</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>2±1</td>
<td>18±5</td>
</tr>
<tr>
<td>8</td>
<td>4</td>
<td>12±7</td>
<td>90±63</td>
</tr>
<tr>
<td>16</td>
<td>8</td>
<td>6±3</td>
<td>183±67</td>
</tr>
<tr>
<td>72</td>
<td>4</td>
<td>4±2</td>
<td>118±31</td>
</tr>
<tr>
<td>120</td>
<td>4</td>
<td>0±0</td>
<td>41±4</td>
</tr>
</tbody>
</table>

P7 rats underwent right carotid artery ligation, followed by exposure to 8% O2. Samples were prepared from the left (“contralateral”) and right (“ipsilateral”) cerebral hemispheres. Panel A data were from animals exposed to 2.5 hours of 8% O2, killed 4 to 120 hours later. Panel B data were from animals exposed to 8% O2 for 1 to 2.5 hours, and killed 16 hours later. Tissue content of MIP-1α was measured using an enzyme-linked immunosorbent assay. Values are expressed as mean ng/g protein±SEM; intergroup differences were evaluated using Kruskal-Wallis tests.

*P<0.05 comparing contralateral hemisphere values.

†P<0.02, comparing ipsilateral hemisphere values.

‡P<0.01, comparing ipsilateral hemisphere values.

Figure 1. NeuN immunohistochemistry identifies neuronal injury. NeuN-immunostained coronal sections at the level of the dorsal hippocampus from normal P7 rat brain (A) and from animals killed 8 (B), 16 (C), 24 (D), 48 (E), or 72 hours (F) after hypoxic-ischemic lesioning. A, Regions where neuronal injury evolves are identified: Ctx indicates cortex; H, hippocampus; Str, striatum; Th, thalamus; Hb, habenula. B, At 8 hours, NeuN staining is reduced in the habenula (arrow) and thalamus (arrowheads). C, At 16 hours, loss of immunostaining extends through ipsilateral cortex (†), striatum (arrow), and hippocampal dentate gyrus (arrowhead). D, At 24 hours, note ipsilateral hemisphere swelling. E, At 48 hours, extensive hippocampal neuronal loss is evident in the pyramidal cell layer (arrowhead) and dentate gyrus (arrow); F, At 72 hours, note hippocampal injury both in the CA1 (arrow) and CA3 (arrowhead) subfields and subtle neuronal loss in the contralateral cortex (†). Scale bar, 1 mm.

Hypoxia-Ischemia (HI) Stimulates MIP-1α Production in Neonatal Rat Brain

A. Time course of HI-induced MIP-1α production

<table>
<thead>
<tr>
<th>Time after HI (h)</th>
<th>N</th>
<th>Contralateral*</th>
<th>Ipsilateral†</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2</td>
<td>0±0</td>
<td>0±0</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>2±1</td>
<td>18±5</td>
</tr>
<tr>
<td>8</td>
<td>4</td>
<td>12±7</td>
<td>90±63</td>
</tr>
<tr>
<td>16</td>
<td>8</td>
<td>6±3</td>
<td>183±67</td>
</tr>
<tr>
<td>72</td>
<td>4</td>
<td>4±2</td>
<td>118±31</td>
</tr>
<tr>
<td>120</td>
<td>4</td>
<td>0±0</td>
<td>41±4</td>
</tr>
</tbody>
</table>

P7 rats underwent right carotid artery ligation, followed by exposure to 8% O2. Samples were prepared from the left (“contralateral”) and right (“ipsilateral”) cerebral hemispheres. Panel A data were from animals exposed to 2.5 hours of 8% O2, killed 4 to 120 hours later. Panel B data were from animals exposed to 8% O2 for 1 to 2.5 hours, and killed 16 hours later. Tissue content of MIP-1α was measured using an enzyme-linked immunosorbent assay. Values are expressed as mean ng/g protein±SEM; intergroup differences were evaluated using Kruskal-Wallis tests.

*P<0.05 comparing contralateral hemisphere values.

†P<0.02, comparing ipsilateral hemisphere values.

‡P<0.01, comparing ipsilateral hemisphere values.
Figure 2. Hypoxia-ischemia stimulates MIP-1α expression. MIP-1α immunostaining at 16 hours after lesioning in the ipsilateral cortex (A), hippocampal dentate gyrus (B), and thalamus (C); there were no immunoreactive cells in corresponding contralateral regions (G through I). In panels C and I, arrowheads identify the third ventricle. Panels D through F highlight specific features of MIP-1α immunostaining: panel D (cortex) illustrates the association of MIP-1α immunoreactive cells with capillaries (arrows); panel E (dentate gyrus) shows a cluster of large, round cells with diffuse homogeneous cytoplasmic MIP-1α immunostaining; and Panel F (thalamus) shows 3 cells with juxtanuclear MIP-1α immunostaining. Scale bars: A, 50 μm (same magnification for B and C, G through I); D through F, 20 μm.

Contralateral hemisphere, much smaller increases in MIP-1α protein were also found (at 16 hours, 6±3 ng/g protein, P<0.05, Kruskal-Wallis test). To estimate the duration of MIP-1α accumulation, samples were also obtained at 72 and 120 hours after lesioning; in the lesioned hemisphere, MIP-1α concentrations remained markedly elevated at 72 hours (118±31 ng/g protein) and declined substantially at 120 hours (5 days after lesioning). Comparison of MIP-1α concentrations attained at 16 hours after hypoxia-ischemia, in animals that underwent durations of hypoxia-ischemia ranging from 1 to 2.5 hours, indicated that MIP-1α concentrations increased with the duration of the insult (P<0.01, Kruskal-Wallis test).

MIP-1α Immunohistochemistry
In normal P7 to 12 rat brain, MIP-1α immunoreactivity was restricted to cells within the corpus callosum, internal and external capsule, and ependyma; findings were similar in animals evaluated 4 hours after lesioning (not shown). At this developmental stage, physiologically activated microglia are concentrated in white matter tracts;* MIP-1α immunostaining in white matter tracts coincided with the distribution of these microglia (see below). In contrast, at 8 hours after hypoxia-ischemia, MIP-1α immunoreactive cells were also widely disseminated throughout the lesioned forebrain, often within or closely associated with capillaries. At 16 hours, MIP-1α immunoreactive cells were readily identified in the parenchyma of the cortex (Figure 2A), hippocampal dentate gyrus (Figure 2B), and habenula and thalamus (Figure 2C); no immunoreactive cells were seen in corresponding contralateral regions (Figure 2G through 2I). In some areas, such as the dentate gyrus of the hippocampus (Figure 2B), immunoreactive cells were found in clusters. Fewer MIP-1α immunoreactive cells were identified in the lesioned anterior striatum at all time points evaluated. No MIP-1α was detected in normal adult brain.

Evaluation at ×40 magnification revealed several distinctive features of MIP-1α immunoreactivity. From 8 to 16 hours, immunoreactive cells were observed closely associated with capillaries (Figure 2D). In addition, 2 distinct patterns of intracellular immunostaining were discernible: a “cytoplasmic” pattern, characterized by homogeneous cellular staining (Figure 2E), and a “juxtanuclear” pattern, characterized by an intense concentration of immunostaining adjacent to the nucleus (Figure 2F). Preliminary analysis suggested that 1 to 5 days after lesioning, the distributions of these 2 MIP-1α staining patterns diverged. To identify possible functional implications of these differences, we systematically compared the neuroanatomic distributions of cytoplasmic and juxtanuclear MIP-1α staining (Figure 3). NeuN and ED-1 immunoreactivity patterns were evaluated concurrently to map the distribution of neuronal injury and monocytic/microglial activation, respectively.
At 8 hours after hypoxia-ischemia, cytoplasmic MIP-1α immunostaining was identified in areas of neuronal injury (Figure 3, first and second column); cytoplasmic MIP-1α peaked at 16 hours and was undetectable at 72 hours. In contrast, juxtanuclear MIP-1α-immunoreactive cells were concentrated in white matter tracts in normal P7 brain (Figure 3, third column), and their distribution coincided with that of physiologically activated microglia (Figure 3, fourth column). At 24 to 72 hours after lesioning, juxtanuclear MIP-1α immunostaining increased throughout the lesion territory and coincided closely with monocyte/microglial infiltration. Of note, at 72 hours after lesioning, there was subtle neuronal injury in the contralateral cortex, and increased juxtanuclear MIP-1α immunostaining and ED-1 immunoreactivity were concentrated in the adjacent white matter. By 5 days (120 hours) after lesioning, juxtanuclear MIP-1α immunoreactivity had waned, whereas ED-1 immunoreactive cells remained diffusely distributed throughout the injured forebrain. The observed decline in MIP-1α immunostaining on P12 was congruent with results of the MIP-1α tissue assays (Table 1).

Immunofluorescence assays were analyzed with confocal microscopy to confirm the cellular sources of MIP-1α (Figure 4). Isolectin B4 labels monocytes and endothelium (Figure 4A). At 8 hours after hypoxia-ischemia, many cells with cytoplasmic MIP-1α immunoreactivity (Figure 4B) were found within lectin-labeled capillaries (Figure 4C). To exclude endothelium as a source of MIP-1α, additional experiments were done in which a von Willebrand factor antibody was used to label endothelium; there was no colocalization of MIP-1α and von Willebrand factor immunoreactivity (not shown).

In contrast, from 24 to 120 hours after lesioning, few MIP-1α immunoreactive cells were found within capillaries. To determine the identity of MIP-1α immunoreactive cells within brain parenchyma, double-labeling assays were performed with antibodies to ED-1, GFAP, and myeloperoxidase (to identify neutrophils). Many MIP-1α-immunoreactive cells expressed ED-1 (Figure 4D through 4F); no MIP-1α immunoreactive cells were identified as astrocytes or neutrophils. Cytoplasmic and juxtanuclear MIP-1α immunostaining patterns were readily identified (Figure 4G and 4I, compared to Figure 2E and 2F), as were cells with both cytoplasmic and juxtanuclear MIP-1α immunoreactivity (Figure 4H).

**CCR1 and CCR5 Immunohistochemistry**

Several cell types expressed the MIP-1α receptors CCR1 and CCR5 in normal P7 to P12 rat brain. Both CCR1 and CCR5 immunoreactivity were localized to cerebral endothelium (Figure 4J and ependyma (not shown). CCR1-immunoreactive astrocytes (GFAP-reactive; Figure 4K) were identified throughout the cortex, thalamus, striatum, and hippocampus. Occasional CCR1-immunoreactive neurons (NeuN-reactive) were identified in the cortex (not shown). Many CCR5-immunoreactive microglia (ED-1 reactive) were identified within the internal and external capsule and in the corpus callosum (Figure 4L). In adult brain, CCR1 and CCR5 immunoreactivity were limited to endothelium and ependyma. CCR1 and CCR5 immunoassays were also performed in sections from lesioned animals; no increases in CCR1 or CCR5 immunoreactivity were detected.

**Discussion**

Our results provide information about MIP-1α, CCR1, and CCR5 expression both in normal and in acutely injured neonatal brain. In normal P7 to P12 rat brain, MIP-1α is expressed in physiologically activated microglia, and its receptors are expressed in multiple cell types, including endothelial cells and ependyma (CCR1 and CCR5), astrocytes and neurons (CCR1), and microglia (CCR5). Their expression patterns at this developmental stage differed from findings in adult rat brain; in the mature brain, there was no MIP-1α immunostaining and CCR1 and CCR5 immunostaining were restricted to endothelium and ependyma.

Hypoxic-ischemic lesioning markedly stimulated expression of MIP-1α for up to 5 days after lesioning; these trends
are congruent with a report of sustained increases in MIP-1α mRNA (up to 72 hours) in this model.7 Immunohistochemistry studies revealed that MIP-1α expression coincided closely with regions where neuronal injury was most pronounced and monocytes/microglia accumulated. Double-labeling experiments demonstrated that MIP-1α immunoreactivity was restricted to cells of the monocyte/macrophage lineage.

Two distinct populations of MIP-1α–immunoreactive cells were identified, based on the cellular distributions of immunostaining. Cells with homogeneous cytoplasmic immunostaining were identified 8 to 48 hours after injury, often near capillaries. A similar distribution of MIP-1α immunostaining was recently reported after middle cerebral artery occlusion in adult rat brain.14 Based on our confocal microscopy results, a substantial fraction of these cells were blood-derived monocytes. Cells with juxtanuclear immunoreactivity were identified both in normal neonatal brain and in the infarct territory; these cells invariably expressed ED-1, an antigen expressed by activated monocytes and microglia. This juxtanuclear cytokine expression pattern, first noted in stimulated lymphocytes,22 reflects concentration of newly synthesized protein within the Golgi apparatus23,24 and indicates that activated monocytes and microglia are sources of MIP-1α in the neonatal brain. We also identified cells with both diffuse cytoplasmic and intense juxtanuclear immunoreactivity, suggesting a continuum between the phenotypes. Of interest, brief exposure of monocytes to stimulatory cytokines in vitro induced diffuse cytoplasmic cytokine immunostaining, whereas more prolonged exposure induced a juxtanuclear expression pattern.23

In this neonatal stroke model, the duration of hypoxia-ischemia determines the severity of tissue injury; the temporal threshold to elicit neuronal injury is about 1.5 hours, and more prolonged insults elicit more widespread injury. Comparison of the tissue concentrations of MIP-1α attained at 16 hours, after hypoxic-ischemic insults of increasing duration (Table 1), demonstrated corresponding trends; MIP-1α production rose with increasing duration of hypoxia-ischemia. Multiple proinflammatory stimuli could regulate MIP-1α production in the acutely injured brain. Two cytokines that are upregulated acutely after neonatal ischemic brain injury, interleukin 1β and tumor necrosis factor-α,5 markedly stimulate MIP-1α mRNA expression in fetal microglia in vitro;25,26 the close association of MIP-1α immunoreactive cells with capillaries at 8 to 16 hours after injury suggests that activated endothelium could be a source of these stimulatory cytokines.27 However, it is likely that other factors regulate the relatively sustained production of MIP-1α within brain parenchyma 1 to 5 days after injury.

Neonatal hypoxic-ischemic brain injury also markedly stimulates production of the β-chemokine MCP-1.24 In the acutely injured neonatal brain, the temporal and anatomic patterns of MIP-1α and MCP-1 expression differ markedly. Most notably, multiple cell types, including neurons, express MCP-1 as early as 4 hours after lesions, whereas MIP-1α expression is restricted to monocytes and microglia. There is substantial evidence that MIP-1α and MCP-1 are differentially regulated in other CNS disorders as well.27 These findings suggest that the 2 chemokines play distinct roles in the inflammatory response to acute brain injury.

Previously, we reported that CCR5 was constitutively expressed in tissue homogenates of P7 rat brain, but we were unable to identify its cellular sources with immunohistochemistry.28 Assay refinements enabled us to identify CCR5 and another MIP-1α receptor, CCR1, on several cell types in neonatal rat brain. The major implication of these findings is that MIP-1α may exert its effects on multiple target sites in the acute post-injury period and that its functions may well extend beyond its best-characterized role as a monocyte chemokine. The relatively sustained increases in MIP-1α, extending up to 5 days after injury, suggest that it could also influence intrinsic repair and recovery mechanisms.

Transient focal ischemia stimulates CCR5 mRNA expression16 in adult rat brain, and excitotoxic injury upregulates hippocampal CCR5 protein expression in neonatal rat brain.28 We did not detect increases in CCR1 or CCR5 immunoreactivity, although many monocytes/microglia and astrocytes accumulated in the lesion territory. One potential explanation for this unexpected finding is that receptor activation and subsequent internalization29 limited detection of these chemokine receptors with immunohistochemistry.

In preliminary experiments, we attempted to delineate the functional role of MIP-1α in the injured neonatal brain by systemic and intracerebral administration of a neutralizing antibody; we did not discern any modulation of tissue injury. However, the technical challenges inherent in performing these studies in neonatal animals may well have restricted our ability to reproducibly deliver adequate concentrations of antibody to the area of injury (R.M. Cowell, BS, et al, unpublished data, 2001).

Conclusion
Our data highlight the prominence of the inflammatory response to acute hypoxic-ischemic brain injury in the neonate and implicate MIP-1α as an important mediator of this response. Yet, the precise pathophysiologic role of MIP-1α in the acutely injured brain remains to be determined. Our findings that both blood-derived monocytes and intrinsic brain microglia express MIP-1α suggest that this chemokine may have complex and distinct effects at different stages of the inflammatory response. In addition, the unexpectedly broad range of cell types that could be responsive to MIP-1α adds to the challenges in elucidating its effects on the progression of acute brain injury.

Acknowledgments
Ms Cowell is the recipient of predoctoral fellowship 9910156Z from the American Heart Association and Dr Silverstein is a recipient of USPHS awards NS31054 and 35059. We would like to thank Thomas Komorowski, University of Michigan Diabetes Research Center, for assistance with confocal microscopy.

References
Chemokines and Hypoxic-Ischemic Brain Injury


Hypoxic-Ischemic Injury Induces Macrophage Inflammatory Protein-1α Expression in Immature Rat Brain
Rita M. Cowell, Haiyan Xu, John M. Galasso and Faye S. Silverstein

Stroke. 2002;33:795-801
doi: 10.1161/hs0302.103740

Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2002 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/33/3/795

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at: http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at: http://stroke.ahajournals.org//subscriptions/