Dendritic Cells Are Present in Ischemic Brain After Permanent Middle Cerebral Artery Occlusion in the Rat

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Background and Purpose—Cerebral ischemia is associated with inflammation involving accumulation of polymorphonuclear neutrophils. T cells have been suggested to contribute to the secondary progression of ischemic brain injury. Dendritic cells (DC) are potent regulators of immunity by activating and tolerizing T cells. DC have previously been detected in rat meninges and choroid plexus. Hypothesizing that DC are involved in inflammation associated with cerebral ischemia, we investigated DC in the brain of Sprague-Dawley rats after permanent middle cerebral artery occlusion (pMCAO) versus sham operation.

Methods—All experimental rats (n=24) had the right MCA permanently occluded by inserting a nylon monofilament through the right external carotid artery. Immunohistochemistry was used to detect DC (OX62⁺), microglia/macrophages (OX42⁺) that developed into DC, and activated DC expressing major histocompatibility complex class II (OX6⁺) in the brain hemispheres at 1 hour to 6 days after pMCAO or sham operation.

Results—Levels of DC were elevated at 1 hour in the ischemic versus sham hemispheres (P<0.001) and ischemic versus nonischemic hemispheres (P<0.0001). Activated DC expressing major histocompatibility complex class II (OX6⁺) were still elevated at 6 days after pMCAO in the ischemic versus nonischemic hemispheres (P<0.01). The area of brain lesion correlated with numbers of OX6⁺ DC per 100-mm² brain tissue section (r=0.79; P<0.0001).

Conclusions—Increased levels of DC in the brain after pMCAO and correlation between DC numbers and brain lesion area indicate a role for DC in cerebral ischemia. This observation could constitute a basis for further studies on the role of DC in inflammation related to cerebral ischemia. (Stroke. 2002;33:1129-1134.)

Key Words: cerebral ischemia, focal cytokines dendritic cells microglia rats

Cerebral ischemia is associated with an intense inflammatory reaction that may contribute to the secondary progression of ischemic brain injury. This inflammation is characterized by accumulation of inflammatory cells and mediators in the ischemic brain.1,2 Leukocytes, mainly polymorphonuclear neutrophils, can be recognized in microvessels as early as 30 minutes after permanent middle cerebral artery occlusion (pMCAO).3 Additionally, other immune cells extrinsic to the brain, such as CD5⁺ T cells, are present after transient MCAO and are increased at the edges of infarcts at 3 to 7 days after pMCAO in the rat.4,5 Increased levels of T cells expressing interleukin (IL)-17, interferon (IFN)-γ, and IL-8 mRNA have been found in the ischemic hemispheres of rats after pMCAO compared with sham-operated animals.6

Several studies indicate a potential role for dendritic cells (DC) in the control of immunity by activating as well as tolerizing T cells.7,8 DC capture and process antigens and then migrate via the blood and afferent lymph to lymphoid organs.9 The central nervous system (CNS) contains few immunocompetent cells because of the anatomic blood-brain barrier but also because of a relative lack of antigen-presenting cells and major histocompatibility complex (MHC) class II molecules.7 OX62, a marker of rat lymph node DC, has been detected in rat meninges and choroid plexus stroma.10 DC present in vessels or parenchyma of the brain may play a role in regulating local inflammation.

We hypothesized that DC may be involved in the inflammation occurring after brain ischemia and evaluated the presence of DC in the brain of Sprague-Dawley rats 1 hour to 6 days after pMCAO versus sham operation.

Materials and Methods
All experiments were performed according to the guidelines issued by the local animal care committee and in compliance with regulations formulated by the Swedish Department of Agriculture. The local animal research committee of Huddinge University Hospital, Karolinska Institutet, approved our procedures (Animal Research Committee Protocol No. S140/98). Forty-eight male Sprague-Dawley rats, weighing 250 to 300 g, were used in this study. Outbred rats were purchased from Charles River Laboratory (Sulzfeld,
Tissue Preparation and Immunohistochemistry

Brains were immediately snap-frozen in liquid nitrogen. Cryostat sections, 10 μm thick, from brains of pMCAO or sham-operated animals were mounted on gelatin-coated glass slides, air dried, and fixed in acetone at −20°C for 5 minutes. Fixed sections were stained with monoclonal antibodies directed against microtubule-associated protein 2 (MAP2) (5 mg/mL, Sigma), OX62 (diluted 1:100, Serotec), OX42 (diluted 1:100, Serotec), and OX6 (diluted 1:100, Serotec) with the use of an avidin-biotin method. The 4 monoclonal antibodies define MAP2, DC, microglia/macrophages, and MHC class II, respectively. All incubations were performed under humidified conditions, and slides were washed 3 times for 5 minutes each in PBS between steps. First, endogenous peroxidase was blocked by incubation for 20 minutes in methanol containing 0.3% hydrogen peroxide. After preabsorption with normal serum, sections were incubated with primary antibody overnight at 4°C. After they were washed, the sections were overlaid for 1 hour with biotinylated horse anti-mouse antibodies (Southern Biotechnology) followed by avidin-biotin complex (ABC Vectastain Elite Kit; Vector). Reactions were visualized. Approximately 17.5 to 18 mm of suture was inserted past the common carotid artery bifurcation to block the origin of the right MCA. For the 24 sham-operated rats, the carotid arteries were exposed, but no suture was inserted. Groups of 4 rats in each group were perfusion-fixed with saline and 4% paraformaldehyde solution and killed with an overdose of pentobarbital at 1, 6, 12, and 24 hours and 2 and 6 days after pMCAO or sham operation. Forebrains were removed for hemisphere dissection at the above times after pMCAO or sham operation.

Combined In Situ Hybridization and Immunohistochemical Staining

To detect mRNA of IL-1β, IL-6, IL-10, IL-12, tumor necrosis factor-α (TNF-α), and IFN-γ, in situ hybridization was performed as described for tissue sections and modified for cell suspensions.12,13 Synthetic oligonucleotide probes (Scandinavian Gene Synthesis AB) were labeled with [35S]deoxyadenosine-5'-α-(thio)-triphosphate (New England Nuclear) with terminal deoxynucleotidyl transferase (Amersham). To increase the sensitivity of the method, a mixture of 3 different oligonucleotide probes (approximately 48 base pairs long) was used. The oligonucleotide sequences were obtained from GenBank, and probes were designed with the use of MacVector software. After emulsion autoradiography, development, and fixation, coded slides were examined by dark field microscopy for positive cells. The intracellular distribution of the grains was always checked by light microscopy at higher magnification. Positive cells always contained ≥15 (usually 50 to 100) grains in a starlike distribution (Figure 1G), while negative cells almost always contained no or very few grains, which were then scattered randomly over the cell and not distributed in a starlike fashion. Consequently, it was only rarely difficult to differentiate between mRNA-positive and negative cells. As control probes, the sense sequence of each cytokine was used in parallel on tissue sections from each animal, without revealing any positive cells. Results were expressed as numbers of labeled cells per 100-mm² tissue section. The tissue section areas were measured by image analysis (Seescan-Image Analysis System).

Figure 1. A, OX622 cells in the ischemic hemisphere at 1 hour after pMCAO (arrow). B, Lack of OX62 cells in the nonischemic hemisphere at 1 hour after pMCAO. C, OX622OX422 cells, ie, microglia/macrophages that had developed into DC in the ischemic hemisphere at 6 days after pMCAO (arrows). D, Low levels of OX622OX422 cells in the nonischemic hemisphere at 6 days after pMCAO. E, Green, red, and orange colors of fluorescence demonstrate OX622 DC, OX68 cells, and OX622OX68 cells, respectively. Arrows indicate higher values, representing activated DC expressing MHC class II in the ischemic hemisphere at 6 days after pMCAO. F, Low levels of OX622OX68 cells in the nonischemic hemisphere at 6 days after pMCAO. Arrow shows OX622OX68 cells expressing IL-1β mRNA in the ischemic hemisphere at 12 hours after pMCAO (arrow). H, Lack of OX62 cells expressing IL-1β mRNA in the nonischemic hemisphere at 12 hours after pMCAO.
and immunohistochemically stained with monoclonal antibody for OX62, as described above. Dehydration in 60%, 95%, and 100% ethanol followed before emulsion.14

Statistical Analysis
Each experimental group consisted of 4 animals. All values are presented as mean±SEM. Differences between the 2 groups were evaluated by Student’s t test.

Results
OX62⁺ and OX62⁺OX6⁺ Cells in Brain Tissue After Brain Ischemia
Immunohistochemistry was used to measure the temporal expression of OX62⁺ DC after pMCAO or sham operation (Figure 2). Very low levels of OX62⁺ cells were detected in animals when examined from 1 hour to 6 days after sham operation. In contrast, numbers of OX62⁺ cells were elevated in the ischemic hemispheres at 1 hour (11-fold increase over sham; \( P<0.001 \)) and at 6 hours (22-fold increase; \( P<0.001 \)), remained elevated at 24 hours, increased at 2 days (25-fold increase; \( P<0.001 \)), and further increased at 6 days after pMCAO (84-fold increase; \( P<0.001 \)). OX62⁺ DC were present in gray matter. They were detected to a greater extent in the lesion periphery and also within the lesion at 1 to 24 hours; at 2 and 6 days OX62⁺ DC were mostly stained within the lesion.

Levels of OX62⁺ DC in the ischemic versus nonischemic hemispheres were also compared (Figure 2). The numbers of OX62⁺ DC were higher in ischemic versus nonischemic hemispheres at 1 hour (93±11 versus 19±2 OX62⁺ cells per 100 mm²; \( P<0.001 \)), gradually were further elevated, and remained elevated with a pronounced difference at 6 days (876±120 versus 73±14 OX62⁺ cells per 100 mm²; \( P<0.001 \)).

Levels of OX62⁺ DC in the nonischemic versus sham hemispheres were also compared. The numbers of OX62⁺ DC were higher in nonischemic versus sham hemispheres at 1 hour (19±2 versus 9±2 OX62⁺ cells per 100 mm²; \( P<0.01 \)), remained elevated for the study period, and differed at 6 days (73±14 versus 11±1 OX62⁺ cells per 100 mm²; \( P<0.01 \)).

Double immunohistochemistry was used to detect DC expressing MHC class II (OX62⁺OX6⁺ cells) in the brain hemispheres at 6 days after pMCAO or sham operation, revealing that OX62⁺OX6⁺ cells were elevated in the ischemic hemispheres at 6 days (4-fold increase over sham; \( P<0.001 \); Figure 3). Numbers of OX62⁺OX6⁺ cells were also higher in ischemic versus nonischemic hemispheres at 6 days (56±3 versus 33±2 OX62⁺OX6⁺ cells per 100 mm²; \( P<0.05 \)).

Microglia in Ischemic Hemispheres Develop Into DC
To investigate whether microglia/macrophages developed into DC, double immunohistochemistry was used with anti-OX62 and anti-OX42 monoclonal antibodies. OX62⁺OX42⁺ cells were investigated in the brain hemispheres from 1 hour to 6 days after pMCAO or sham operation (Figure 4). Numbers of OX62⁺OX42⁺ cells were slightly higher in ischemic hemispheres at 1 hour (3-fold increase over sham), significantly elevated at 6 hours (23-fold increase; \( P<0.01 \)), remained elevated at 24 hours and at 2 days (11-fold increase; \( P<0.01 \)), and increased further at 6 days after pMCAO (24-fold increase; \( P<0.001 \)). Numbers of OX62⁺OX42⁺ cells were also higher in the ischemic versus nonischemic hemispheres (Figure 4) at 1 hour (10±2 versus 6±1 OX62⁺OX42⁺ cells per 100 mm²) and were significantly elevated from 24 hours to 6 days after pMCAO, with a pronounced difference at 6 days (300±29 versus 13±4 OX62⁺OX42⁺ cells per 100 mm²; \( P<0.0001 \)).

Expression of DC in Ischemic Hemispheres Correlates With Area of Brain Lesion
Areas of tissue necrosis were plotted from projections of the coronal sections. The area of tissue necrosis was divided by the total area of the whole-brain coronal section as measured by image analysis. The area of brain lesion (percentage)
Ischemic Neuronal Damage and DC Expressing IL-1β, IL-6, IL-10, IL-12, TNF-α, and IFN-γ RNA in Brain Tissue After Brain Ischemia

Ischemic neuronal damage, detected as loss of MAP2 immunoreactivity, expanded in the ischemic hemispheres at 6 hours and reached a peak at 24 hours in the ischemic versus sham hemispheres (33±3 versus 71±3 MAP2 mRNA–expressing cells per 100 mm²; \( P<0.0001 \)). The levels of MAP2 mRNA–expressing cells decreased in the ischemic hemispheres at 2 and 6 days after pMCAO. The sham-operated rats showed MAP2 mRNA expression to a similar extent from 1 hour to 6 days after surgery. Immunohistochemical staining for DC with OX62 was combined with in situ hybridization to measure expression of IL-1β, IL-6, IL-10, IL-12, TNF-α, and IFN-γ mRNA over time after pMCAO or sham operation. Figure 6 shows the temporal profiles of mRNA expression for these molecules in brain sections from ischemic and nonischemic hemispheres from animals subjected to pMCAO and from sham-operated control animals.

IL-1β mRNA–expressing DC (Figure 6A) were present in the ischemic hemisphere at 1 hour and were elevated over sham at 6 hours (2-fold increase over sham; \( P<0.01 \)) and at 12 hours (5-fold increase; \( P<0.01 \)), then decreased and were not different compared with sham at 24 hours and 2 and 6 days after pMCAO. IL-1β mRNA–expressing DC (Figure 6A) were also higher in ischemic versus nonischemic hemispheres at 6 hours (when mean values were 13±1 versus 6±1 IL-1β mRNA–expressing DC per 100 mm²; \( P<0.01 \)), further increased at 12 hours (27±5 versus 5±1 IL-1β mRNA–expressing DC per 100 mm²; \( P<0.01 \)), and decreased at 24 hours to 6 days after pMCAO.

IL-12 mRNA–expressing DC (Figure 6B) were elevated in the ischemic versus sham hemispheres at 1 hour (3-fold increase; \( P<0.01 \)), remained elevated from 6 hours to 2 days, and were still elevated at 6 days (3-fold increase; \( P<0.01 \)). IL-12 mRNA–expressing DC (Figure 6B) were also higher in the ischemic versus nonischemic hemispheres at 6 hours (9±1 versus 5±1 IL-12 mRNA–expressing DC per 100 mm²; \( P<0.05 \)) and were markedly increased at 6 days (14±2 versus 4±1 IL-12 mRNA–expressing DC per 100 mm²; \( P<0.001 \)).

Likewise, IL-6 mRNA–expressing DC (Figure 6C) were elevated in the ischemic versus sham hemispheres at 1 hour (3-fold increase; \( P<0.01 \)), remained elevated 2 days after pMCAO, but did not differ from sham at 6 days. IL-6 mRNA–expressing DC (Figure 6C) were also higher in the ischemic versus nonischemic hemispheres at 12 hours (13±2 versus 5±1 IL-6 mRNA–expressing DC per 100 mm²; \( P<0.05 \)) and then decreased to similar levels at 6 days.

IL-10 mRNA–expressing DC (Figure 6D) were elevated in the ischemic versus sham hemispheres at 1 hour (3-fold increase; \( P<0.05 \)), increased at 24 hours (4-fold increase; \( P<0.001 \)), remained elevated at 2 days, and decreased to sham levels at 6 days after pMCAO. IL-10 mRNA–expressing DC (Figure 6D) were also higher in the ischemic versus nonischemic hemispheres at 24 hours (19±2 versus 6±2 IL-10 mRNA–expressing DC per 100 mm²; \( P<0.01 \)), remained higher at 2 days (12±1 versus 5±1 IL-10 mRNA–expressing DC per 100 mm²; \( P<0.001 \)), but did not differ at 6 days after pMCAO.

TNF-α mRNA–expressing DC (Figure 6E) were detectable in the ischemic hemispheres at 1 hour and were elevated versus sham at 6 hours (4-fold increase; \( P<0.01 \)), remained higher versus sham at 12 and 24 hours, and increased at 2 and 6 days (3-fold increase; \( P<0.05 \)). TNF-α mRNA–expressing DC (Figure 6E) were also higher in the ischemic versus nonischemic hemispheres at 6 hours (14±2 versus 8±2 TNF-α mRNA–expressing DC per 100 mm²; \( P<0.05 \)), remained higher at 12 hours, did not differ at 24 hours, and then increased again at 6 days (17±3 versus 9±1 TNF-α mRNA–expressing DC per 100 mm²; \( P<0.05 \)).

IFN-γ mRNA–expressing DC (Figure 6F) were detectable in the ischemic hemisphere at 1 hour, were elevated versus sham at 24 hours (4-fold increase; \( P<0.01 \)), and further increased at 2 and 6 days (7-fold increase; \( P<0.0001 \)) after pMCAO. IFN-γ mRNA–expressing DC (Figure 6F) were also higher in the ischemic versus nonischemic hemispheres at 24 hours (10±2 versus 5±1 IFN-γ mRNA–expressing DC per 100 mm²; \( P<0.05 \)) and were even higher at 6 days (20±2 versus 8±1 IFN-γ mRNA–expressing DC per 100 mm²; \( P<0.001 \)) after pMCAO.

Discussion

Brain ischemia is characterized by local inflammation reflected by accumulation of inflammatory cells and of a multitude of mediators that may potentiate the ischemic CNS injury. Identification of mediators involved in the selective recruitment and accumulation of inflammatory cells in the...
ischemic brain may have an impact on therapy. To our knowledge, there are no previous data on DC in cerebral ischemia. In this study we demonstrate increased numbers of DC in ischemic compared with sham hemispheres as early as 1 hour after pMCAO \( (P<0.001) \), with further increase \( (P<0.001) \) at 6 days. Our data also confirm that microglia may develop into DC, as reflected by an increase of OX62/\( \text{OX42} \) cells in the ischemic hemispheres. Previous studies indicate that glial cells exposed to granulocyte-macrophage colony-stimulating factor (GM-CSF) may turn into DC-like cells.\(^{15}\) (B-G. Xiao, PhD, et al, unpublished data, 2000). Consequently, the DC that are observed in the brain parenchyma may be of local origin and develop into DC on endogenous stimulation by GM-CSF, though we cannot rule out the possibility of peripherally derived DC. Interestingly, the numbers of DC in the brain correlated with the size of the brain lesion during the observation time from 1 hour to 6 days after pMCAO \( (r=0.79; \ P<0.0001) \), further supporting a role for DC in cerebral ischemia. DC express several cytokines, such as IL-1β and IL-6. Levels of DC expressing IL-1β and IL-6 mRNA were increased at 12 hours; this therefore occurred before maximal neuronal damage at 24 hours, as reflected by loss of MAP2 mRNA expression. IL-1β and IL-6 may play a role in the expansion of the ischemic injury. Additionally, other cytokines, such as TNF-α, have been extensively investigated in cerebral ischemia. Exogenous administration of TNF-α exacerbates focal ischemic injury,\(^{16}\) whereas neuronal damage caused by focal cerebral ischemia is exacerbated in TNF-receptor knockout mice, indicating that TNF-α may play a neuroprotective role.\(^{17}\) Our results with a biphasic profile of DC expressing TNF-α in the ischemic hemispheres may indicate that TNF-α may assert deleterious and/or beneficent effects in a time-dependent way.

IFN-γ, which is produced by T cells and not by resident CNS cells, increases the expression of MHC class II molecules, which are important for antigen presentation, further supporting a role for IFN-γ in brain necrosis. We have previously shown increased levels of IFN-γ mRNA expressing blood mononuclear cells and secretion of IFN-γ at 12 hours after pMCAO, which thus occurs before increased levels in the CNS; this supports primary systemic involvement of IFN-γ that, at later stages, may affect the CNS injury.\(^{6}\) This hypothesis is supported by present data in which

Figure 6. Temporal profiles of numbers of DC expressing IL-1β, IL-12, IL-6, IL-10, TNF-α, and IFN-γ mRNA in ischemic, nonischemic, and sham hemisphere samples at 1, 6, 12, and 24 hours and 2 and 6 days after pMCAO or sham operation. *\( P<0.05, **P<0.01, ***P<0.0001, \) ischemic versus nonischemic hemispheres.
levels of DC expressing IFN-γ are increased in the ischemic hemispheres at 6 days after pMCAO. Further functional studies on IFN-γ involvement in cerebral ischemia are warranted.

The function of DC in mediating inflammatory responses in the CNS is incompletely understood. McMenamin found high levels of MHC class II* (OX6*) DC in dura mater, leptomeninges, and choroid plexus of the rat, i.e., at sites that may encounter CNS antigens. In the present study OX62+OX6 cells were elevated in the ischemic versus sham hemispheres at 6 days after pMCAO (P<0.0001). Tissue injury can lead to activation of DC. Hypothetically, as a consequence of brain ischemia, DC may capture and process antigens from damaged or necrotic tissue, migrate to lymphoid organs, and activate T cells. These activated T cells can pass the blood-brain barrier, which becomes leaky as a consequence of CNS injury, infiltrate into the CNS, and contribute to the inflammation secondary to brain ischemia.

DC may not only initiate immune responses resulting from antigen presentation but can also affect immune responses by producing cytokines, as supported by this study, and in other ways. For example, DC produce high levels of nitric oxide, which promotes apoptosis of autoreactive T cells as well as of DC themselves, thereby ameliorating experimental allergic encephalomyelitis. Suppression of experimental allergic encephalomyelitis by nasal administration of both IL-4 and TGF-beta mRNA in the ischemic hemispheres at 6 days after pMCAO, which supports the involvement of T cells in the inflammation secondary to brain ischemia.

In summary, our findings of increased levels of DC in the brain after pMCAO, as well as of a positive correlation between DC numbers and the area of brain infarct, indicate a role for DC in cerebral ischemia. DC also express several cytokines. Further studies of DC in experimental stroke are needed to extend our knowledge of their role in the inflammation accompanying cerebral ischemia.

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References
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