Lack of Interleukin-6 Expression Is Not Protective Against Focal Central Nervous System Ischemia

Wayne M. Clark, MD; Lisa G. Rinker, BS; Nikola S. Lessov, MD; Kristin Hazel, BA; Jennifer K. Hill, BS; Mary Stenzel-Poore, PhD; Felix Eckenstein, PhD

Background and Purpose—Interleukin-6 (IL-6) appears to be involved in the inflammatory response associated with central nervous system (CNS) ischemia. Although IL-6 levels increase after stroke, it is not known whether IL-6 directly influences CNS ischemic injury. In this study, we used a focal reversible stroke model to investigate whether mice lacking IL-6 were protected against acute ischemic injury.

Methods—We bred IL-6-deficient C57 black mice (I-129 IL-6 KO back-crossed with C57), including homozygous knockouts (IL-6 \(-/-\)), heterozygous littermates (IL-6 \(+/-\)), and normal littermates (IL-6 \(+/+\)). The status of all animals was confirmed by DNA sampling and polymerase chain reaction analysis. Reversible middle cerebral artery occlusion was produced by advancing a silicone-coated 8-0 filament into the internal carotid artery for 2 hours (experiment 1) or 45 minutes (experiment 2). At 24 hours, animals were evaluated on a 28-point clinical scale, blood and cerebrospinal fluid were obtained, and the brains were evaluated for infarct volume and IL-6 mRNA levels.

Results—In experiment 1 (severe ischemia), no differences were seen in lesion size or neurological function between the groups: lesion volume was IL-6 \(-/-\) (n=15), 57±13 mm\(^3\); IL-6 \(+/-\) (n=15), 58±23 mm\(^3\); and IL-6 \(+/+\) (n=15), 58±18 mm\(^3\) (P=NS). ELISA testing confirmed very low to absent levels of IL-6 in the serum and cerebrospinal fluid of knockout animals. Brain mRNA levels of the other proinflammatory cytokines, including tumor necrosis factor-\(\alpha\), IL-1\(\beta\), and IL-1 receptor antagonist, were 50% lower in IL-6-deficient ischemic animals than in normal animals. In experiment 2 (mild ischemia), no differences were seen in lesion size or neurological function between the groups: lesion volume was IL-6 \(-/-\) (n=10), 16±8 mm\(^3\); IL-6 \(+/-\) (n=10), 14±4 mm\(^3\); and IL-6 \(+/+\) (n=10), 19±12 mm\(^3\) (P=NS).

Conclusions—In this study, infarct size and neurological function at 24 hours were not different in animals deficient in IL-6 after transient CNS ischemia. This suggests that IL-6 does not have a direct influence on acute ischemic injury. Further study investigating the role of IL-6 on long-term recovery after stroke is in progress. (Stroke. 2000;31:1715-1720.)

Key Words: inflammation ■ interleukins ■ stroke ■ mice, knockout

There is increasing evidence that the inflammatory response plays an important role in the potentiation of central nervous system (CNS) ischemic injury.\(^1\) Much of this inflammatory response appears to be mediated by interleukins (ILs), a multifunctional subclass of cytokines.\(^2\) The proinflammatory interleukins, including IL-1, tumor necrosis factor (TNF)-\(\alpha\), and IL-6, can influence the function and synthesis of other cytokines by a complex cytokine network.\(^2\) These proinflammatory interleukins are produced by a variety of cells, including microglial cells, astrocytes, and leukocytes, and appear to directly modulate CNS cell apoptosis, differentiation, and proliferation. Cytokines may also be involved in the activation and recruitment of leukocytes into the CNS. IL-1\(\beta\), TNF-\(\alpha\), and IL-6 have been shown to activate leukocytes and increase the expression of adhesion receptors on leukocytes (CD-18), endothelial cells, and astrocytes (intercellular adhesion molecule-1). Several investigators have characterized the role of IL-1 and TNF-\(\alpha\) in experimental CNS ischemia and have found a therapeutic benefit of IL-1 receptor antagonist (IL-1RA) treatment.\(^3\)\(^-\)\(^7\)

The role of IL-6 in CNS ischemia has been studied less widely. Indirect evidence for the involvement of IL-6 in ischemic injury comes from clinical studies that found that cerebrospinal fluid (CSF) and plasma levels of IL-6 predict the functional recovery of the patient and correlate with the infarct size.\(^8\)\(^-\)\(^12\) IL-6 mRNA has also been shown to be upregulated after focal CNS ischemia.\(^13\) Other studies have suggested that IL-6 mRNA expression is highest in ischemia-
Materials and Methods

All animal procedures were approved by the Oregon Health Sciences University Institutional Review Board and are in accordance with guidelines published by the National Institutes of Health for animal use. Because the inflammatory response appears to be involved predominantly in reperfusion injury, we chose a reversible focal CNS ischemic model: the filament middle cerebral artery occlusion (MCAO) model with reperfusion. In the first experiment, we used the MCAO model to investigate the effect of IL-6 expression on severe CNS ischemic injury with reperfusion. Two hours of MCAO was used, because this duration of ischemia has previously been found to produce a consistent large area of severe ischemic injury involving half of the hemisphere. In the second experiment, we chose a shorter duration of ischemia, 45 minutes, in an effort to investigate IL-6 effects in milder ischemic conditions. We have previously found that this duration of ischemia produces a stroke of milder ischemic injury involving <20% of the hemisphere.

IL-6–Knockout Breeding

IL-6–knockout animals were bred as follows. IL-6–knockout (−/−) male I-129 mice (B6129-ILKO:TM1J)) were purchased from Jackson Laboratory, Bar Harbor, Me. In these mice, transcriptional mRNA for IL-6 is present; however, the mechanism for translation is deleted at the second exon so that no IL-6 protein appears in CSF or serum. This is a selective deletion such that the translation of the other cytokines is not affected (data from Jackson Laboratory). We have previously found that focal CNS ischemia is not reliably produced in I-129 animals with the MCAO model. We therefore back-crossed the I-129 IL-6–deficient animal with C57 black mice (C57BL/6N), a strain that does produce reliable CNS infarcts. Homozygous knockout offspring of the I-129 and C57 pairing were back-crossed with normal C57 animals for a total of 6 generations. At each point, the knockout status of the animals was confirmed by tail DNA sampling with polymerase chain reaction analysis. At this point, we observed reliable CNS infarcts in the animals, and colony breeding was started with heterozygous pairs. This pairing allows for the production of homozygous IL-6–knockout animals (−/−) along with litter-matched heterozygous IL-6–knockout animals (−/+), and normal controls (+/+). The IL-6–knockout mice exhibit no overt phenotypes compared with normal C57 mice. However, they tended to show decreased exploratory activity. This increase in fear-related behavior has been reported in IL-6–knockout animals.

Experimental Design

In experiment 1 (severe ischemia), 45 male mice weighing 26 to 36 g were used. The 30 mice consisted of 10 homozygous IL-6–knockouts (IL-6−/−), 10 heterozygous littermates (IL-6−/+), and 10 normal littermates (IL-6+/+). All procedures were the same as in experiment 1 except that the animals were subjected to only 45 minutes of MCA ischemia before the surgery was removed.

Sample Collection

At 24 hours after reperfusion, the animals were rated on a 28-point neurological scale for focal deficits by 2 investigators (see Table 1). At the time of death, the animal was anesthetized, and CSF was obtained via suboccipital puncture with a 27-gauge needle. CSF was collected and immediately placed in dry ice. The still anesthetized animal was then decapitated, and ~1 mL of blood was collected. The blood was allowed to clot for 2 hours before being centrifuged for 20 minutes at 2000g. Approximately 300 μL of serum was collected and frozen on dry ice. In experiment 1, the brain was quickly removed and embedded in a warm bed of agarose on a Stoelting tissue slicer, by which a 1-mm coronal section of tissue (fourth section from the rostral end of the brain) was removed from the center of the brain for histological analysis. The rostral and caudal sections of the brain were then divided by left and right hemispheres and placed in tubes in powdered dry ice for mRNA analysis. All samples except the 1-mm brain section for histology were stored at −80°C until ready for use. In experiment 2, the entire brain was sliced into six 1-mm sections for lesion volume assessment.

Histology

In experiment 1, the 1-mm tissue section was fixed in 10% formalin for 24 hours and then placed in 15% sucrose. Once the section sank to the bottom of the well plate, the 15% sucrose solution was replaced by 30% sucrose containing 0.05% sodium azide and stored at 4°C until sectioning occurred. The 1-mm section was mounted on a freezing microtome and was embedded in OCT Tissue-Tek (No. 4583, Miles Inc). Frozen tissue was cut into 50-μm sections. Approximately 10 good sections were obtained, and 2 of these were mounted on chrome-albumin-jelly slides. Slides were dried at room temperature overnight and then baked in a 37°C oven for a minimum of 6 hours. Slides were then immersed in a 50%/50% chloroform/ethanol 37°C bath for a minimum of 2 hours to delipidize the tissue. Sections were stained with Luxal fast blue (ICN Biomedicals Inc) and counterstained with cresyl violet acetate (Eastman Kodak Co) to differentiate between the area of ischemia and unaffected tissue. In experiment 2, the six 1-mm sections were placed into 2% 2,3,5-triphenyltetrazolium chloride (TTC) in 0.9% PBS and stained at 37°C for 30 minutes. After staining, the TTC was replaced with 10% phosphate-buffered formalin. Areas not stained red with TTC were considered lesions and were used to calculate lesion areas.

Image Analysis

The NIH Image Analysis program in conjunction with a 1200–dots per inch flatbed scanner was used to measure the area of ischemia and total hemisphere area (ipsilateral and contralateral). Slides were...
placed on a scanner bed, and an image of each section was captured and saved for later analysis. Two investigators independently imaged the scanned sections, and the areas were averaged. In experiment 1, estimated volumes, in cubic millimeters, were calculated by use of the 0.5-mm slice thickness and the sum of the 2 measured areas, multiplied by a predetermined factor previously calculated to estimate total lesion volume using a center slice area ($R^2=0.96$ with actual total infarct volume). This was done to estimate the lesion volume in the tissue used for mRNA analysis. In experiment 2, volumes were determined by use of slice thickness and the measured areas of the lesion and ipsilateral hemisphere.

**ELISA**

To confirm that IL-6 was not being produced in plasma and CSF in knockout animals, a sandwich enzyme immunoassay technique was applied by use of a Quantikine mouse IL-6 kit from R & D Systems (experiment 1 only). All serum samples were diluted 1:2 according to kit instructions and run on 96-well plates with 2 wells per sample (50 μL/well). The CSF samples (6 μL) were diluted to 50 μL. The ELISA was performed with 2 polyvinyl 96-well plates coated with polyclonal antibody specific for mouse IL-6 according to the manufacturer’s specifications. The well plates were then read on a Molecular Devices Kinetic microplate reader with Softmax software at a wavelength of 450 nm and correction wavelength of 540 nm.

**Ribonuclease Protection Assay**

To determine whether the absence of IL-6 expression would influence the expression of other proinflammatory cytokines known to be important in CNS ischemia, we measured brain levels of IL-1β, TNF-α, and IL-1RA using ribonuclease protection assay (experiment 1 only). The total RNA was prepared from frozen brain tissue samples by use of Tri-Reagent (Molecular Research Center, Inc). Left and right hemispheres from each subject were homogenized in 5.5 mL of Tri-Reagent with an IKA Labotechnik homogenizer. Total RNA was extracted in chloroform, ethanol-precipitated, and frozen at −80°C. All samples were then read on a spectrophotometer (260 and 280 nm) to determine concentration and purity. These samples were then brought to the same concentration, and pools of RNA were made from group and condition (eg, stroke IL-6 −/−, control IL-6 −/−, etc).

A RiboQuant Multi-Probe RNAase Protection Assay kit (Pharmingen) containing a total of 17 cytokine probes, including IL-1RA, IL-1β, and TNF-α, was used to analyze mRNA. Templates of distinct sizes were used to generate a [32P]-labeled antisense RNA probe set and hybridized in excess to target RNA. Total RNA (10 μg) was hybridized (56°C, 18 hours) with the labeled probe set, followed by RNase treatment and analyses of protected bands on a denaturing 5% polyacrylamide gel. Gels were subjected to Phosphorimage analysis, and bands were quantified by densitometry with the NIH Image Analysis program, allowing a comparison of each cytokine band to a known housekeeping band (L32). This method produces quantifiable mRNA unit values that can be compared with each other within an experiment. However, the units cannot be expressed in any standardized external value.

**Statistics**

An ANOVA was used to assess the statistical significance of differences in lesion volume and cytokine values between groups. Corrected unpaired t tests were used to assess individual between-group differences. Between-group differences in neurological function were assessed by nonparametric testing.

**Results**

**Experiment 1 (2 Hours of Ischemia)**

A total of 45 animals were evaluated, 15 in each genotype group. There was no difference in temperature between the groups at any time point. All animals had successful MCAO confirmed by laser Doppler: baseline blood flow in the MCA was IL-6 −/−, 387±131 perfusion units (pu); IL-6 +/+ , 380±133 pu; and IL-6 +/+, 396±140 pu ($P=NS$). All postocclusion (5 minute) values were <10%: IL-6 −/−, 24±11 pu; IL-6 +/+, 26±13 pu; and IL-6 +/+, 23±14 pu ($P=NS$). Overall, all ischemic groups had consistent large infarcts with very small SDs for this model. The results of the infarct area (center slice), calculated volume analyses, and functional outcome are summarized in Table 2. There was a correlation of 0.841 between calculated infarct volume and functional outcome. Calculated lesion volume at 24 hours was IL-6 −/−, 57±13 mm$^3$ (50±12% of hemisphere); IL-6 +/−, 58±23 mm$^3$ (48±18%); and IL-6 +/+, 58±18 mm$^3$ (52±14%) ($P=NS$). Neurological function score [mean (range)] was IL-6 −/−, 14.5 (9 to 25.5); IL-6 +/+, 14.5 (11.5 to 20.5); IL-6 +/+, 15 (9 to 26.5) ($P=NS$). Serum IL-6 levels (ELISA) at 24 hours were IL-6 −/−, 2±3 pg/mL; IL-6 +/−, 314±119* pg/mL; IL-6 +/+, 337±131* pg/mL. CSF IL-6 levels at 24 hours were IL-6 −/−, 4±12 pg/mL; IL-6 +/−, 36±40* pg/mL; IL-6 +/+, 67±69* pg/mL ($^*P<0.05$ compared with IL-6 −/−). Ribonuclease protection assay

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**TABLE 1.  Focal Deficits (0–28)**

<table>
<thead>
<tr>
<th></th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Body symmetry (open bench top)</td>
<td>Normal</td>
<td>Slight asymmetry</td>
<td>Moderate asymmetry</td>
<td>Prominent asymmetry</td>
<td>Extreme asymmetry</td>
</tr>
<tr>
<td>2 Gait (open bench top)</td>
<td>Normal</td>
<td>Stiff, inflexible</td>
<td>Holds onto slope, does not slip or climb</td>
<td>Limping</td>
<td>Slides down slope, unsuccessful effort to prevent fall</td>
</tr>
<tr>
<td>3 Climbing (gripping surface, 45° angle)</td>
<td>Normal</td>
<td>Climbs with strain, limb weakness present</td>
<td>Slides immediately, no effort to prevent fall</td>
<td>Slides immediately, no effort to prevent fall</td>
<td>Does not walk</td>
</tr>
<tr>
<td>4 Circling behavior (open bench top)</td>
<td>Not present</td>
<td>Predominantly 1-sided turns</td>
<td>Circles to 1 side (not constantly)</td>
<td>Circles to 1 side</td>
<td>Pivoting, swaying, or no movement</td>
</tr>
<tr>
<td>5 Front limb symmetry (mouse suspended by its tail)</td>
<td>Normal</td>
<td>Light asymmetry</td>
<td>Marked asymmetry</td>
<td>Prominent asymmetry</td>
<td>Slight asymmetry, no body/limb movement</td>
</tr>
<tr>
<td>6 Compulsory circling (front limbs on bench, rear suspended by tail)</td>
<td>Not present</td>
<td>Tendency to turn to 1 side</td>
<td>Circles to 1 side</td>
<td>Pivots to 1 side sluggishly</td>
<td>Does not advance</td>
</tr>
<tr>
<td>7 Whisker response (light touch from behind)</td>
<td>Symmetrical response</td>
<td>Prominent asymmetry</td>
<td>Absent response ipsilaterally, diminished contralaterally</td>
<td>Absent proprioceptive response bilaterally</td>
<td></td>
</tr>
</tbody>
</table>
was performed to determine how the lack of IL-6 influenced the expression of IL-1β, TNF-α, and IL-1RA mRNA levels in the brain. The results for the 3 cytokine brain mRNA levels are shown in the Figure for each of the genotype groups. Each data point represents pooled data from the 15 animals. Across genotype groups, levels of each of the 3 cytokines were higher in the ischemic hemispheres than in controls irrespective of genotype: IL-1β, P = 0.009; IL-1RA, P < 0.001; TNF-α, P = 0.008. Within the transgenic groups for all of the cytokines, the mRNA levels in the ischemic IL-6−/− knockout animals were significantly lower (∼50%) than in normal-type ischemic animals (P = 0.03 overall ANOVA; P = 0.001 IL-6−/− versus IL-6 +/+); see Figure.

**Experiment 2 (45 Minutes of Ischemia)**

A total of 30 animals were evaluated at 24 hours, 10 in each genotype group. There was no difference across groups at any time point. All animals had successful MCAO confirmed by laser Doppler: baseline blood flow in the MCA was IL-6−/−, 378±160 pu; IL-6 +/+; 381±160 pu; IL-6−/−, 403±160 pu (P = NS). All postocclusion (5 minute) values were <10%; IL-6−/−, 26±13 pu; IL-6−/−, 27±11 pu; IL-6−/−, 25±13 pu (P = NS). In this experiment, the laser Doppler was left in place, and values were obtained 10 minutes after the suture was removed to confirm successful reperfusion: IL-6−/−, 366±240 pu; IL-6−/−, 381±136 pu; IL-6−/−, 415±174 pu (P = NS). The results of the volume analyses and functional outcomes are summarized in Table 2. We have also included infarct area (center slice) and what the corresponding calculated infarct volume would be to allow comparisons to the 2-hour group results. All 45-minute groups had consistent small to medium-size infarcts, although the variability was higher than in the 2-hour-ischemia group. Lesion volume at 24 hours was IL-6−/−, 17±10 mm³ (16±8%); IL-6−/−, 15±4 mm³ (14±4%); IL-6−/−, 21±14 mm³ (19±12%) (P = NS). Neurological function score [median (range)] was IL-6−/−, 9 (6 to 12.5); IL-6−/−, 10 (5 to 12.5); IL-6−/−, 9 (6.5 to 12.5) (P = NS).

**Discussion**

This study found that after stroke, the neurological function and CNS injury seen in animals deficient in IL-6 was no different from those in littermate controls. This was true both in animals with severe ischemia and in groups subjected to milder ischemia. These results suggest that IL-6 does not have a direct influence on acute CNS injury related to ischemia. All animals in the present study were evaluated at 24 hours. As such, these results represent early ischemic injury events, including cerebral edema, and do not address whether IL-6 has a role in later recovery from stroke. Further study involving evaluations at later time periods (1 month) are needed to answer this question.

This lack of IL-6 effect on acute stroke injury and function is somewhat surprising, given the strong association between clinical stroke recovery, infarct size, and risk of recurrent stroke. However, all of these previous clinical studies reported on correlations between IL-6 and various stroke outcomes and did not attempt to directly modulate IL-6 levels. It is possible that in the acute phase, IL-6 is merely a marker of the extent of underlying injury.

Using ELISA testing, we confirmed the absence of IL-6 in the serum and CSF of IL-6−/− animals (probably zero, given test sensitivity and variability). The increased levels of IL-6 in serum and CSF at 24 hours in the heterozygous and normal animals are similar to those seen in our previous experimental studies. In these studies, peak levels of IL-6 after MCA occlusion actually occur at ∼6 hours (∼1200 pg/ml), with the 24-hour value being ∼25% of the peak. It appears that both the normal and heterozygous animals had similar IL-6 release, suggesting that the heterozygous animals had sufficient IL-6 mRNA translation for normal cytokine production. A potential weakness of this study is that because
neuronal cell death in cultures. 31 Although these studies have shown early neurodegenerative changes, 23,24 IL-6 also appears to contribute to the reactive gliosis that appears after CNS ischemic injury. Injection of IL-1Ra, and TNF-a mRNA at 24 hours in focal ischemia is consistent with our previous studies and others. 2,8,10,26 We previously found that peak brain cytokine mRNA levels in the mouse MCAO model occur at 24 hours. 15,21 Although this is the first study to directly investigate the effect of IL-6 in stroke, the modulation of other proinflammatory cytokines has produced changes in CNS ischemic injury. Injection of IL-1ß into the lateral ventricle during transient ischemia produced an increase in the infarct size, whereas injection of an anti–IL-1 mononclonal antibody produced a decrease in postischemic infarct size. 27 Treatment with IL-1Ra injected directly into the brain also decreased focal experimental CNS ischemic injury, associated cerebral edema, and neutrophil infiltration into the ischemic areas. 28,29 Finally, Relton et al 30 found that in a rat MCAO model, peripherally administered IL-1Ra significantly reduced infarct size and associated cerebral edema. No transgenic studies have been reported with IL-1. Studies involving TNF-a have found that local injection of TNF-a into the brain greatly increased leukocyte infiltration to this area, suggesting that TNF-a may potentiate CNS reperfusion injury. 2 Other studies have found that TNF-a promotes neuronal cell death in cultures. 31 Although these studies suggest that TNF-a is detrimental during CNS ischemia, a conflicting view has recently been reported. Using TNF receptor knockout (TNFR-KO) mice, Bruce et al 32 found that animals without TNF-a had larger infarcts (MCAO) and lower neuronal survival than control animals.

No previous studies have been reported in IL-6–knockout animals with CNS ischemia. However, IL-6–knockouts have been used in other disease models. Alonzi et al 33 used an IL-6–knockout mouse in an autoimmune arthritis model and found that animals lacking IL-6 were protected against all arthritis-related damage, including the presence of inflammatory cells. In another study, knockout IL-6 animals were found to have a decreased inflammatory response to LPS. 34 These results, along with our findings of decreased expression of proinflammatory cytokines in the brain of IL-6–knockouts, suggest that IL-6–deficient animals do have a decreased inflammatory response. In our study, however, this decreased inflammatory response was not associated with a reduction in neurological ischemic injury.

Conclusions
This study found that the response to either severe or moderate CNS ischemic injury was the same in animals lacking IL-6 as in matched controls. At 24 hours after reversible focal ischemia, both the extent of CNS injury and functional outcome were similar in all groups. These results suggest that strategies targeting inhibition of IL-6 during stroke are unlikely to prove beneficial in the short-term recovery phase. Further studies investigating the role of IL-6 in long-term recovery after stroke are in progress.

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References
Inflammation has long been recognized to be a component process in the complex series of events that accompany acute infarction of the brain. A substantial body of evidence has accrued which suggests that activation of various cytokines occurs as a result of acute stroke, but the problem has been in discerning whether cytokine activation is a part of the problem, exacerbating ischemic damage, or part of the solution, helping to clean up the mess.

In the accompanying article, Clark and colleagues hypothesize that if the proinflammatory cytokine IL-6 were exacerbating ischemic brain injury, then “knockout” animals genetically incapable of producing IL-6 should have smaller infarcts when subjected to the same ischemic injury as IL-6–producing controls. Their data convincingly show that in either mild or severe focal ischemic injury, there was no significant difference in infarct size at 24 hours regardless of IL-6 status, thereby exonerating IL-6 as a “bad actor” in the early infarct scenario. Whether there may be longer-term influences is the subject of ongoing research.

These observations suggest that blockade of IL-6 activity may not be a useful therapeutic target for acute ischemic stroke. The jury is still out on the other elements of the inflammatory cascade, but perhaps this study may serve as a model to guide future research on which factors are of greatest importance in this complicated, multifaceted problem.

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