Effect of neutralization of rat interleukin 6 bioactivity on inducible nitric oxide synthase up-regulation and cerebral ischemic damage

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Objectives: Our aim was to investigate whether neutralization of rat interleukin 6 (IL-6) bioactivity attenuates inducible nitric oxide synthase (iNOS) up-regulation and ameliorates cerebral ischemic damage in a model of focal central nervous system (CNS) ischemia.

Methods: Seventy rats were randomly allocated to groups: Group I (n=10) consisted of normal controls; Group II (n=20) underwent surgical exposure of the middle cerebral artery but no cauterization; the remaining 40 rats were subjected to middle cerebral artery occlusion. Immediately after occlusion, each of these 40 rats was randomly assigned to either the occlusion-only group (Group III, n=20) or the occlusion plus IL-6 antibody treatment group (Group IV, n=20). Half of the rats from each of Groups II, III and IV were euthanized at 24 hours and the other half at 72 hours. The samples were used for iNOS immunohistochemistry and structural analysis.

Results: A single dose of the antibody had no effect on structural changes and iNOS at 24 hours after occlusion. However, administering three doses of the antibody resulted in markedly decreased quantitative and qualitative levels of iNOS-positive stained cells and milder subcellular damage compared with the findings in the occlusion-only group at 72 hours after occlusion.

Discussion: Our findings prove that IL-6 bioactivity is one of the pathological events that trigger the induction of iNOS in the process of CNS ischemic injury. It appears that there may be therapeutic value in neutralization of IL-6 bioactivity to attenuate iNOS up-regulation and ameliorate cerebral ischemic damage in long-term recovery. [Neurol Res 2008; 000: 000–000]

Keywords: Cerebral ischemia; inducible nitric oxide synthase; interleukin 6; rat

INTRODUCTION
Interleukin 6 (IL-6) has been shown to be up-regulated in ischemic cortex after focal cerebral ischemia¹. IL-6, which is one of the main inflammation-associated cytokines, is produced by a variety of cells in the central nervous system (CNS)². Cytokines are recognized mediators of inflammatory responses³. Inflammatory cells secrete these substances, some of which induce inducible nitric oxide synthase (iNOS) expression⁴. iNOS contributes to ischemic brain injury, leading to neurotoxicity⁵–¹⁰.

In the present work, our aim was to investigate whether neutralization of rat IL-6 bioactivity attenuates iNOS up-regulation and ameliorates cerebral ischemic damage in a model of focal CNS ischemia.

MATERIAL AND METHODS
Protocol
Seventy adult male Wistar rats weighing 280–310 g were used in this work. The animals were randomly allocated to groups: Group I (n=10) consisted of normal controls; Group II (sham-operated, n=20) underwent surgical exposure of the middle cerebral artery (MCA) but no cauterization; the remaining 40 rats were subjected to middle cerebral artery occlusion (MCAO). Immediately after occlusion, each of these 40 rats was randomly assigned to either the occlusion-only group (Group III, n=20) or the occlusion plus IL-6 antibody treatment group (Group IV, n=20). All evaluations were performed in blinded fashion.

Surgical procedures
All animals were anesthetized with an intramuscular injection of ketamine (50 mg/kg) and xylazine (10 mg/kg). MCAO was performed using a technique that was initially introduced by Tamura et al.¹¹ but modified by Menzies et al.¹². All surgical procedures were done with the aid of an operating microscope. The right MCA trunk was exposed via a right subtemporal craniectomy using a saline-cooled dental drill. The dura mater was then carefully incised and reflected. Three to 6 mm of the main MCA in the region between the origin of the lateral striate artery and the inferior cerebral vein was
cauterized using bipolar forceps under the surgical microscope. The wound was closed in layers.

Group I (the controls) was used to determine normal brain structure and iNOS immunoreactivity in the absence of surgery or medication. Group II (sham-operated) underwent craniectomy and received an intraperitoneal injection of saline equal in volume to the injection of anti-rat IL-6 given to the antibody-treated group. The aim in this work was to determine the impact of craniectomy alone on iNOS immunoreactivity and brain structure. Group III (occlusion only) received an intraperitoneal saline injection identical to that described above immediately after MCAO. Group IV (anti-rat IL-6 antibody-treated) received one intraperitoneal injection of anti-rat IL-6 antibody (5 μg) (Ig class: recombinant rat IL-6-specific goat IgG, catalog number AF506; R&D Systems Biotech Division, Minneapolis, MN, USA) immediately after occlusion, a second dose (5 μg) 24 hours later and a third dose (5 μg) at 48 hours post-occlusion.

It was necessary to take account of a number of factors in determining the dosage and administration of the antibody. Recent experimental works have demonstrated that peak serum and cerebrospinal fluid (CSF) levels (~1200 pg/ml) of IL-6 after MCAO in mice occur at ~6 hours, with the 24-hour value being close to 25% of the maximum. The exact concentration of antibody required to neutralize rat IL-6 activity is dependent on the IL-6 concentration. However, the exact IL-6 concentration in the rat CNS after occlusion is not known. To provide a guideline, R&D Systems has determined the neutralization dose 50 (ND50) for this antibody under a specific set of conditions. The ND50 for this antibody is defined as the concentration of antibody required to yield one-half maximal inhibition of IL-6 activity on a responsive cell line. The ND50 of anti-rat IL-6 antibody was determined to be 0.03–0.09 μg/ml in the presence of 0.6 ng/ml of recombinant rat IL-6 (R&D Systems). Accordingly, the ND50 of the anti-rat IL-6 antibody used in this work was determined to be ~5 μg based on previous reports as well as the rat's body weight and blood and CSF volume. As detailed, we used a dose of 5 μg at each injection in the treated rats.

Sample collection
Half of the rats from each of Groups II, III and IV were eternized at 24 hours and the other half at 72 hours after craniectomy alone (Group II) or occlusion (Groups III and IV). This created two subgroups per group, with Groups A-II, A-III and A-IV eternized at 24 hours and Groups B-II, B-III and B-IV at 72 hours. In Group IV, the ten rats that were eternized at 24 hours post-occlusion received one dose, and the other ten that were eternized at 72 hours received three doses.

Each rat was transcardially perfused immediately after it was anesthetized. The initial perfusion was done with ~100 ml of 0.1 M phosphate-buffered saline (PBS, pH 7.3). Then, 150 ml of 4% paraformaldehyde was infused under constant pressure using an infusion pump. Ten minutes after perfusion, each brain was removed. Tissues for the assays were taken from the ischemic core, and these included areas of second- and third-degree ischemia, as defined by Menzies et al. The sample from the ischemic core was divided coronally into two consecutive blocks that were assigned numbers, counting from anterior to posterior. The first block was used for iNOS immunohistochemistry and the second for structural analysis.

Light and electron microscopy
Tissues for electron microscopic examination were immediately placed in 5% glutaraldehyde buffered at pH 7.4 with Milloning’s phosphate buffer for 3 hours. Next, the samples were fixed in 1% osmic acid for 2 hours. They were then dehydrated in graded ethanol baths, embedded in araldite and processed for transmission electron microscopy using conventional methods.

For light microscopic examination, the specimens were fixed in 10% neutral formaldehyde solution and embedded in paraffin. Sections, 4–6 μm thick, were cut and stained with hematoxylin–eosin and acid fuchsin/cresyl violet. Slides were examined under a Nikon Optiphot trinocular light microscope.

iNOS immunohistochemistry
Upon removal from the animal, the tissue blocks designated for immunohistochrometry were placed in the buffered neutral formalin and left overnight in a refrigerator at 4°C. The next day, the tissue samples were dehydrated in graded ethanol baths, embedded in paraffin and sliced in 4–6-μm-thick sections. The sections were deparaffinized with 100% xylene and rehydrated in graded ethanol baths. They were then incubated in 3% H2O2 to block endogenous peroxidase activity and washed in PBS. Next, the sections were incubated for 10 minutes in a serum-blocking solution to block non-specific binding. Thereafter, they were incubated overnight at 4°C with anti-iNOS antibody (SA-200; Biomol, Hamburg, Germany). Finally, we used the streptavidin–biotin technique for visualization and processed the sections with a Zymed kit (cat. no. 95-6143; Zymed Lab, San Francisco, CA, USA). For negative controls, some of the brain sections were treated with a non-immune serum instead of the same concentration of primary antibody. Aminoethylcarbazole was used as the chromogenic substrate.

Immunohistochemical staining for iNOS was scored using a microscopic immunohistochemistry grading system scale to quantify iNOS-positive stained cells. iNOS-positive stained cells were counted at a magnification of ×200. At this magnification, the field examined encompassed an area of 2.54 mm². The histological slides were first scanned at low magnification, and the area of maximum iNOS-positive stained cellular density (IPSCD) was selected for grading. We defined the IPSCD as the highest number of iNOS-positive stained cells per field (×200) encountered in the brain sections. Ten fields in the most iNOS-positive stained cellular area of the brain sections were counted.
and only the one having the highest IPSCD was used for analysis.

The Mann–Whitney U test was used for all statistical analyses.

RESULTS

Immunohistochemical examination

As expected, there was no iNOS staining in the negative controls because these tissues were treated with non-immune serum instead of primary antibody. In Group I, mononuclear cells of the pia mater and some microglial cells showed iNOS immunoreactivity. Similar immunostaining was observed in Groups A-II and B-II. In Group A-III, in addition to mononuclear cells in the pia–arachnoid membranes and microglial cells, some of the neurons in the cortex also showed iNOS immunoreactivity. The iNOS staining in Group A-IV was similar to that in Group A-III. In Group B-III, the inflammatory cells showed strong immunoreactivity to anti-iNOS antibody (Figure 1). In addition, some of the neurons of the cortex and hippocampus stained positive for iNOS. Group B-IV showed weak immunoreactivity to anti-iNOS antibody in the neuronal and microglial cells (Figure 2). Table 1 summarizes the qualitative scoring conducted according to the level of iNOS-positive staining observed in the control, sham-operated, occlusion-only and anti-rat IL-6 antibody-treated groups.

A summary of semi-quantitative data on the number of iNOS-positive stained cells observed in the groups is given in Figure 3. The number of iNOS-positive cells in the sham-operated groups did not differ statistically from the number in the control group (p>0.05). Ischemic injury alone resulted in a markedly increased number of iNOS-positive cells compared with the controls and the sham-operated groups at both time points post-occlusion (p<0.05). In Group B-IV, anti-rat IL-6 antibody treatment significantly attenuated the increase in the number of iNOS-positive cells that was observed in Group B-III (p<0.05). In Group A-IV, anti-rat IL-6 antibody treatment attenuated the increase in the number that occurred in Group A-III, but this difference was not significant (p>0.05).

Histological and ultrastructural findings

Group I: The light and electron microscopic findings for the neuronal and glial cells and capillary wall were normal.

Groups A-II and B-II: Mild cellular changes were seen in these sham-operated groups, but the ultrastructure of the neuronal cells, glial cells and capillaries was normal.

Group A-III. The most prominent light microscopic findings were interstitial and intracellular edema, necrosis and hemorrhage. There was also significant perivascular and perineuronal edema (Figure 4). Electron microscopic study of the neurons revealed nuclear heterochromatin clumping, increased cytoplasmic density and swelling of the mitochondria and endoplasmic reticulum cisternae. As observed on light microscopy, the neuronal cytoplasm was hyperchromatic with evidence of nuclear membrane alterations. The perikaryon of the neurons showed varying degrees of shrinkage, disorganization and vacuolation. The microglia were activated, with the expression of iNOS staining being prominent (Figure 5). The astrocyte reaction was observed in the perivascular area and the neuropil. The capillaries showed a marked increase in the number of red blood cells, with some dilated capillaries and apparent red cell extravasation. The pericyte reaction was prominent, with strong immunoreactivity to anti-iNOS antibody in the perivascular area. The endothelial cells were also positive for iNOS staining, with strong immunoreactivity to anti-iNOS antibody in the perivascular area. The endothelial cells were also positive for iNOS staining, with strong immunoreactivity to anti-iNOS antibody in the perivascular area.

Table 1: iNOS immunoreactivity in rat brains from the different experimental groups

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<th>Group</th>
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+, positive staining in mononuclear cells in the pia–arachnoid membranes and some microglial cells; ++, positive staining in mononuclear cells in the pia–arachnoid membranes but weak immunoreactivity in the neuronal and microglial cells; ++++, in addition to mononuclear cells in the pia–arachnoid membranes and microglial cells, positive staining in some neurons, and particularly prominent staining in inflammatory cells.

Figure 1: Group B-III: The submeningeal inflammatory cells (arrows), neurons (arrowheads) and microglia (m) show positive iNOS staining. Aminoethylcarbazole and hematoxylin, original magnification ×200

Figure 2: Group B-IV: iNOS staining was prominent in the meningeal cells (arrows), but the neurons (arrowheads) and microglia (m) showed only minimal immunoreactivity. Aminoethylcarbazole and hematoxylin, original magnification ×200

Table 1: iNOS immunoreactivity in rat brains from the different experimental groups
microscopy, many areas showed perineuronal edema (Figure 5). There were also minor ultrastructural changes in the glial cells and capillary endothelial disruption and vacuolization of cytoplasmic organelles.

**Group A-IV.** Edema, hemorrhage and necrosis were also seen in this group. Neuronal shrinkage and dark neuron change were observed in most areas. There was the fine ultrastructure of the neuronal and glial cells and nerve fibers. The nerve cells exhibited mitochondrial vacuolization, disruption of mitochondrial cristae and enlargement of the endoplasmic reticulum and Golgi apparatus cisternae. The myelinated nerve fibers showed axonal and myelin sheath degeneration. Many of the glial cells exhibited mild to moderate ultrastructural changes, but some areas showed pericapillary edema.

**Group B-III.** The light microscopic findings of this group were interstitial and intracellular edema, necrosis and hemorrhage. There was also significant perivascular and perineuronal edema. Regions of neuronal necrosis and edema were prominent. The nerve cell ultrastructure showed clumping of nuclear heterochromatin, mitochondrial swelling and disintegration of the mitochondrial cristae. The oligodendrocytes showed vacuolization of the mitochondria and the endoplasmic reticulum cisternae. The myelinated nerve fibers exhibited myelin sheath degeneration and axonal changes. The capillary endothelial cells showed mild ultrastructural changes, including clumping of nuclear heterochromatin and increased electron density in the
cytoplasm. In addition, there was marked pericapillary edema (Figure 7).

Group B-IV. The light microscopic findings for the neuronal cells, glial cells and capillary walls were moderate edema, necrosis and hemorrhage (Figure 8). Electron microscopic examination revealed mild to moderate neuronal and glial cell changes, including nuclear chromatin clumping and disruption of cytoplasmic organelles. Although the myelinated nerve fibers exhibited slight myelin sheath changes, the axonal ultrastructure was normal. The capillary wall ultrastructure was normal, but there were areas of pericapillary edema (Figure 9).

DISCUSSION

iNOS is not detected in healthy tissue. When substrates or cofactors for iNOS become limiting, cytotoxic end-products are produced. Induction of iNOS is most striking in immunocompetent cells that have been exposed to cytokines. Microglia are the immunocompetent cells in the CNS and are known to express iNOS when subjected to adequate stimulation. In addition to microglial cells, iNOS expression in the CNS increases under pathological conditions when macrophages, neurons, astrocytes, vascular smooth-muscle cells and endothelial cells are exposed to cytokines in vitro. Block et al. demonstrated that 1 day after MCAO in rats there were several IL-6-positive microglia and neurons in the ischemic penumbra, and this expression of IL-6 increased on day 3 and remained elevated up to day 14. Gijbels et al. also reported that systemically administered IL-6 antibody penetrates the CSF in animals with non-infectious CNS inflammatory disease, hampering the development of the normally induced disease process. It is believed that applying anti-serum that has been raised against a particular hormone or chemical compound can neutralize the function of that compound in vivo. These data were our basis for using anti-IL-6 antibody.

Widenka et al. found iNOS labeling in endothelial cells, vascular smooth-muscle cells and adventitial cells at 7 days after experimental subarachnoid hemorrhage. They also noted some staining for iNOS in activated microglia, glial networks and neurons. Sayama et al. reported that on days 1 and 2 after experimental subarachnoid hemorrhage, some neutrophils and mononuclear cells in the brain tissue were positive for iNOS antibody. Wada et al. claimed that the major cellular...
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sources of iNOS expression after traumatic brain injury were astrocytes and macrophages. Gahm et al.27 demonstrated in vivo induction of iNOS only in neutrophils and macrophages after moderate focal brain trauma.

In our work, control rats exhibited iNOS immunoreactivity in the mononuclear cells of the pia–arachnoid membranes and microglial cells in the brain. These tissues and cells did not stain positive in the control samples of previous immunohistochemical investigations23,24,27, and we have no definitive explanation for this discrepancy. Higher sensitivity of our testing method may be one reason for the difference. In addition, our results indicated increased expression of iNOS in mononuclear cells in the pia–arachnoid membranes, microglial cells, inflammatory cells and some neurons in regions of the cortex and hippocampus after occlusion. This indicates that our antibody and immunohistochemistry protocol was sensitive enough to detect very small changes in iNOS immunoreactivity.

Regarding time-related changes observed after brain trauma, some reports have stated that iNOS is not involved in the early cell changes because up-regulation requires relatively long survival (2–3 days) after ischemic injury28,29. Disputing this, in their experiments on rat brain, ladecola et al.7 found that iNOS and catalytic activity were detectable 12 hours after cerebral ischemia and that they peaked at 48 hours and then returned to baseline after 7 days. Widenka et al.24 reported that immunohistochemical staining for iNOS was markedly intense in rat brain 7 days after experimental subarachnoid hemorrhage. Gahm et al.27 reported that cerebral iNOS was expressed in a delayed manner, beginning 6 hours after experimental brain contusion, with a peak at 24 and 48 hours. Wada et al.26 found there was a tendency toward elevated iNOS activity at 1 day after head injury. They noted significantly elevated levels at 3 days and a peak at 7 days. They also reported that 3 days of treatment with the iNOS inhibitor aminoguanidine led to a decrease in total cortical necrotic neuron counts.

In our work, ischemic injury alone led to a marked increase in quantity of iNOS-positive cells at specific points in time post-occlusion in the MCAO-only groups. Specifically, we found markedly increased expression of iNOS in mononuclear cells in the pia–arachnoid membranes, in microglial cells, in some neurons in regions of the cortex and in inflammatory cells at 24 hours in the MCAO-only group. In addition to these cells, some hippocampal neurons also exhibited over-expression of iNOS at 72 hours in this group. These results concur with those of previous works28,29. In addition to the significant increase at quantitative and qualitative levels of iNOS-positive stained cells, light and electron microscopy revealed marked structural changes in the ischemic core after occlusion only.

In the treatment groups, a single dose of anti-rat IL-6 antibody had no effect on ischemic injury-induced structural changes and quantitative–qualitative levels of iNOS-positive stained cells in the ischemic core when testing was done 24 hours after occlusion. However, administering three doses of anti-rat IL-6 antibody resulted in markedly decreased quantitative and qualitative levels of iNOS-positive cells and milder subcellular damage and less edema compared with the findings in the occlusion-only group when testing was done 72 hours post-occlusion. These immunohistochemical changes also correlated well with the structural changes in the brain tissues at 72 hours post-occlusion. It seems that the single dose of antibody was ineffective at neutralizing IL-6 and indirectly attenuating iNOS induction in the ischemic core. On the other hand, multiple doses of anti-rat IL-6 antibody neutralize IL-6 bioactivity and indirectly attenuate iNOS induction and thus partially block the progression of edematous and structural changes in the ischemic brain tissue. This is in agreement with a previous finding that various nitric oxide synthase inhibitors act in a dose-dependent manner to reduce neurotoxicity in vivo settings30.

CONCLUSIONS

Our findings prove that IL-6 bioactivity is one of the pathological events that trigger the induction of iNOS in the process of CNS ischemic injury. Taken together, it appears that there may be therapeutic value in neutralization of IL-6 bioactivity to attenuate iNOS up-regulation and ameliorate cerebral ischemic damage in long-term recovery.

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